Litton BIONETICS

SCREENING STUDIES
CONTRACT FDA 71-268
COMPOUND FDA 71-56 a.1-14
PROPYLENE GLYCOL
HOST-MEDIATED ASSAY
CYTOGENETICS
DOMINANT LETHAL ASSAY
N24

Summary of mutagenicity screening studies-Contract FDA 71-268 & Compound FDA 71-56 host-mediated assay cytogenetics dominant lethal assay-Propylene Glycol
3/5/74

N25

LBI PROJECT #2446

SUMMARY OF MUTAGENICITY SCREENING STUDIES

CONTRACT FDA 71-268
COMPOUND FDA 71-56 9-7-74
PROPYLENE GLYCOL
HOST-MEDIATED ASSAY
CYTOGENETICS
DOMINANT LETHAL ASSAY

SUBMITTED TO

FOOD & DRUG ADMINISTRATION
DEPARTMENT OF HEALTH, EDUCATION AND WELFARE
ROCKVILLE, MARYLAND

SUBMITTED BY

LITTON BIONETICS, INC.
7315 WISCONSIN AVENUE
BETHESDA, MARYLAND

MARCH 5, 1974

Litton BIONETICS

Litton BIONETICS

7315 Wisconsin Avenue, Bethesda, Maryland 20014 301 652-6616

April 11, 1974

Mr. William L. Totten
Contracting Officer
Negotiated Contracts Branch
Division of Contracts & Grants Management
Food & Drug Administration, HFA-510
5600 Fishers Lane, Room 4C-25
Rockville, Maryland 20852

Reference: Contract FDA 71-268; LBI's Project #2446

Dear Mr. Totten:

Litton Bionetics, Inc. is pleased to submit a report for the referenced contract entitled "Mutagenicity Screening Studies" for compound FDA 71-56, Propylene Glycol.

Included in this report are the results and raw data of the three tests conducted: The Host-Mediated Assay; the Cytogenetic Studies; and the Dominant Lethal Assay. Three (3) copies are being submitted for your review.

If there are any questions concerning this report, or, if additional information is required, please do not hesitate to contact me.

Sincerely,

LITTON BIONETICS, INC.

Robert J. Weir, Ph.D.
Vice President

RJW:lls
Enclosures (3)

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I. REPORT

A. Introduction

Litton Bionetics, Inc. (LBI) has investigated the possible mutagenicity of compounds selected and provided by the Food and Drug Administration under Contract 71-268. LBI's investigation utilized the three mammalian test systems herein described -- Host-Mediated Assay, Cytogenetic Studies and Dominant Lethal Assay. These tests provide information as to the types of genetic damage caused by environmental compounds -- pesticides, chemicals, food additives, drugs and cosmetics.

The Host-Mediated Assay is based upon the assumption that the action of a mutagen on the genetics of bacteria is similar to that in man. This is further strengthened by the use of an eukaryotic organism (Saccharomyces cerevisiae). Since the mutation frequencies are well established for the indicator organism, any deviation due to the action of the test compound is readily detectable. As some compounds are mutagenic in bacteria and not in the host animal, and vice versa, this test is able to differentiate an action which may have been due to hosts' ability to detoxify or potentiate a suspected mutagen. This action is dependent upon the ability of the compound to gain access to the peritoneal cavity. Coupled with the direct action of the compound on the indicator organism in vitro, the assay provides a clear insight into host-mediation of mutagenicity.

Cytogenetics provides a valuable tool for the direct observation of chromosomal damage in somatic cells. Alteration of the chromosome number and/or form in somatic cells may be an index of mutation. These studies utilized examination of bone marrow cells arrested in C-metaphase from rats exposed to the test compound as compared to positive and negative control animals. If mutational

changes occur, the types of damage expected due to the action of chemicals are structural rearrangements, breaks and other forms of damage to the chromosomal complement of the cells exposed.

For the in vitro cytogenetic studies, we have a more rapid and inexpensive means of determining chromosomal damage. This is accomplished by observing cells in anaphase. As the chromatids separate and move along the spindle, aberrations may occur. Chromatids which do not migrate to the daughter cells may lead to uneven distribution of parts or of entire chromatids (mitotic nondysjunction). These give rise to "side arm" bridges which have been interpreted as point stickiness or localized failures of chromosome duplication point errors. These aberrations (bridges, pseudochiasmata, multipolar cells, acentric fragments, etc.) are extremely sensitive indicators of genetic damage.

The Dominant Lethal Test is an accurate and sensitive measure of the amount and type of fetal wastage which may occur following administration of a potential mutagen. Dominant lethal mutations are indicators of lethal genetic lesions. The effects of mutagens on the chromosomal complement of the spermatozoa of treated males results in alterations of form and number of chromosomes. Structural rearrangements and aneuploidy may lead to the production of non-viable zygotes, early and late fetal deaths, abortions and congenital malformations. In addition, aberrations could lead to sterility or reduced reproductive capacity of the F_1 generation. The action of a mutagen on specific portions of spermatogenesis is also apparent in this test.

B. Objective

The purpose of these studies is to determine any mutagenic effect of the test compound by employing the Host-Mediated Assay, Cytogenetic Studies

and the Dominant Lethal Assay, both in vivo and in vitro tests are employed with the cytogenetic and microbial test systems. These tests and their descriptions are referenced in the Appendices A through F.

C. Compound

1. Test Material

Compound FDA 71-56, Propylene Glycol, Lot #YA07102A, as supplied by the Food and Drug Administration.

2. Dosages

The animals employed, the determination of the dosage levels and the route of administration are contained in the technical discussion.

The dosage levels employed for compound FDA 71-56 are as follows for Cytogenetics Studies in vivo in rats.

Low Level	30	mg/kg
Intermediate Level	2500	mg/kg
LD_5	5000	mg/kg
Negative Control	saline	
Positive Control (TEM*)	0.3	mg/kg

The dosage levels employed for compound FDA 71-56 are as follows for Host-Mediated Assay in vivo in mice.

Low Level	30	mg/kg
Intermediate Level	2500	mg/kg
LD_5	5000	mg/kg
Negative Control	saline	
Positive Control (EMS**)	350	mg/kg
(DMN***)	100	mg/kg

* Triethylene Melamine
** Ethyl Methane Sulfonate
*** Dimethyl Nitrosamine

The dosage levels employed for compound FDA 71-56 are as follows for the Dominant Lethal Assay *in vivo* in rats.

Low Level	30	mg/kg
Intermediate Level	2500	mg/kg
LD_5	5000	mg/kg
Negative Control	saline	
Positive Control (TEM*)	0.3	mg/kg

The *in vitro* cytogenetics studies were performed employing three logarithmic dose levels.

Low Level	0.001	mcg/ml
Medium Level	0.01	mcg/ml
High Level	0.1	mcg/ml
Negative Control	saline	
Positive Control (TEM*)	0.1	mcg/ml

*Triethylene Melamine

The discussion of this test is contained in the technical discussion.

D. Methods

The protocols employed are explained in Appendices C and D.

E. Summary

1. Host-Mediated Assay

This compound caused no significant increases in mutant frequencies with *Salmonella* TA-1530 and with all levels with *Salmonella* G-46 except the acute high level which may be a weak or questionable positive. *Saccharomyces* D-3 showed increased recombinant frequencies at all levels except the acute high dose. Subacute studies produced increased recombinant frequencies at all levels. Recoveries of this organism decreased markedly as the compound increased. *In vitro* *Salmonella* tests were negative and *Saccharomyces* tests were positive.

2. Cytogenetics

a. *In vivo* - The compound produced no detectable significant aberration of the bone marrow metaphase chromosomes of rats when

administered orally at the dosage levels employed in this study.

b. In vitro - The compound produced no significant aberration in the anaphase chromosomes of human tissue culture cells when tested at the dosage levels employed in this study.

3. Dominant Lethal Assay

This compound was considered to be non-mutagenic in rats in the Dominant Lethal Assay when using the dosages employed in this study.

F. Results and Discussion

1. Toxicity

a. In vivo

A group of ten (10) male rats with an average body weight of 291.2 was given compound FDA 71-56. The compound was in a solution of 0.85% saline and was administered by gastric intubation at a single dose of 10,000 mg/kg of body weight. The animals received 4 ml of the compound on the first day of study. All animals appeared normal during treatment and for an additional six (6) days post-treatment observation. Necropsies of these animals were performed on day seven (7) and revealed no gross morphological changes in the organs examined.

b. In vitro

WI-38 cells in each tube were administered the concentrations of compound listed below. The cells were observed for the presence of CPE and mitoses.

Tube No.	No. of Cells	Conc. mcg/ml	CPE	Mitoses
1	$5X10^5$	0.1	-	+
2	$5X10^5$	0.1	-	+
3	$5X10^5$	1.0	-	+
4	$5X10^5$	1.0	+	-
5	$5X10^5$	10.0	+	-
6	$5X10^5$	10.0	+	-
7	$5X10^5$	100.0	+	-
8	$5X10^5$	100.0	+	-
9	$5X10^5$	1000.0	+	-
10	$5X10^5$	1000.0	+	-

A closer range finding was determined as follows.

Tube No.	No. of Cells	Conc. mcg/ml	CPE	Mitoses
1	$5X10^5$	0.1	-	+
2	$5X10^5$	0.1	-	+
3	$5X10^5$	0.25	-	$\pm$
4	$5X10^5$	0.25	+	-
5	$5X10^5$	0.50	+	-
6	$5X10^5$	0.50	+	-
7	$5X10^5$	0.75	+	-
8	$5X10^5$	0.75	+	-
9	$5X10^5$	1.0	+	-
10	$5X10^5$	1.0	+	-

The high level employed was 0.1 mcg/ml, the intermediate level was 0.01 mcg/ml, and the low level was 0.001 mcg/ml.

c. TOXICITY DATA SHEETS

CONTRACT FDA 71-268

COMPOUND FDA 71-56

PROPYLENE GLYCOL

TOXICITY DATA

CONTRACT FDA 71-268

COMPOUND FDA 71-56

PROPYLENE GLYCOL

Solvent:	0.85% Saline
Dosage Form:	Solution
Animals:	Male rats with an average body weight of 291.2 grams for each dosage group. All animals were observed for seven (7) days.
LD_{50}:	Could not be determined at dosages up through 10 grams per kilogram. Therefore, the dosages selected for the mutagenicity studies were:

High Level	5,000 mg/kg
Intermediate Level	2,500 mg/kg
Low Level	30 mg/kg

The LD_{50} is greater than 10 grams per kilogram and there was no abnormal gross pathology on the animals used in this study.

2. Host-Mediated Assay

Compound FDA 71-56 produced no significant changes in the mutant frequencies in tests with Salmonella TA-1530. With Salmonella G-46 all dose levels were negative except the acute high level which is a weak or questionable positive.

Tests with Saccharomyces D-3 resulted in a dose response increase at the acute low and intermediate dose levels. The high level caused a low recombinant frequency (below the negative control) which may well have been due to selective killing of the mutants. The total recoveries decreased as the dosage increased. The recoveries in the acute high group were about 1/3 that of the control. Subacute studies produced increased recombinant frequencies at all levels. The recoveries were severely depressed and it appears that the recombinant frequencies were depressed to even a higher relative degree. The data are difficult to interpret, and at the dose levels used it appears the compound may be recombinogenic.

a. EVALUATION SHEET

Compound: 71-56 Propylene Glycol

Indicator Strain	In Vitro	In Vivo: Possible Low Recoveries	In Vivo: Controls	Other Comments
TA-1530 NC,PC,Acutes (9/29/72) Subacutes	pos. (neg.)	NC PC AL AI AH SANC SAL (SAI) SAH	NC OK PC OK ~~SANC-OK~~	1. All doses negative 2. SAI recovery 1.22: freq. 2.02. Not out of line - should accept.
G-46 9/15/72 All	pos. (neg.)	NC PC AL AI AH SANC SAL SAI SAH	NC OK PC OK ~~SANC--OK~~	1. SAH dose may be positive but weak. All animals in the test look reasonable therefore can't make absolute decision.
D3 8/25/72 All	(pos.) neg.	NC 2.37 PC 24.97 AL 12.5* AI 23.89 (AH) 4.13** SANC SAL 10.12 SAI 7.99 (SAH) 10.19**	NC low PC low ~~SANC-~~	1. Controls: extremely high recoveries depressed the normal freq. of 5.0 to about 1/2 value in NC. Positive control depressed similarly. 2. AL and AI doses are positive AH dose negative. Compound appears to affect organism viability. 3. S-acute doses also appear positive. Seems to be less effect on organisms here.

* 4 animals have 1 or less recomb.
** recovery low and yeast appear to be killed

Summary: Results for TA-1530 and G-46 appear acceptable. Interpretation of the D3 results is complex. There does appear to be killing at both high doses in D3 which might make definite assessment of positive or negative difficult. The low and intermed. doses for the acutes and s-acutes are probably positive. I would guess that the AL doses would have been as high as the AI if most of the animals had responded in a uniform way. The results are not easily interpreted and I would hesitate to call the data accptable. However, a reapeat at the same dose levels would probably not generate anything better. See compd. 71-28.

David Brusick

b. HOST-MEDIATED ASSAY SUMMARY SHEETS

CONTRACT FDA 71-268

COMPOUND FDA 71-56

PROPYLENE GLYCOL

HOST MEDIATED ASSAY

SUMMARY SHEET

OUTLIERS REMOVED

COMPOUND: FDA 71-56

	SALMONELLA TA1530		SALMONELLA G-46		SACCHAROMYCES D-3	
	MMF (X 10E-8)	MFT/MFC	MMF (X 10E-8)	MFT/MFC	MRF (X 10E-5)	MRT/MRC
ACUTE						
NC	.99		.97		2.04	
PC	9.33	9.42	16.96	17.48	23.53	11.53
AL	1.01	1.02	.50	.52	9.41	4.61
AI	1.37	1.38	1.09	1.12	23.89	11.71
AH	.75	.76	.69	.71	4.13	2.02
SUBACUTE						
NC	.99		.97		2.04	
SL	.79	.80	1.08	1.11	10.12	4.96
SI	1.53	1.55	1.73	1.78	6.91	3.39
SH	2.07	2.09	3.50	3.61	5.62	2.75

IN VITRO	TA1530	G-46	D-3 % CONC	D-3 % SURVIVAL	D-3 R X 10E5
NC					
PC					

STOP

HOST MEDIATED ASSAY

SUMMARY SHEET

OUTLIERS INCLUDED

COMPOUND: FDA 71-56

	SALMONELLA TA1530		SALMONELLA G-46		SACCHAROMYCES D-3	
	MMF (X 10E-8)	MFT/MFC	MMF (X 10E-8)	MFT/MFC	MRF (X 10E-5)	MRT/MRC
ACUTE						
NC	.99		.97		2.37	
PC	9.33	9.42	16.96	17.48	24.97	10.54
AL	1.01	1.02	.50	.52	12.50	5.27
AI	1.67	1.69	1.09	1.12	23.89	10.08
AH	1.08	1.09	.88	.91	4.13	1.74
SUBACUTE						
NC	.99		.97		2.37	
SL	1.13	1.14	1.08	1.11	10.12	4.27
SI	2.02	2.04	1.73	1.78	7.99	3.37
SH	2.07	2.09	3.50	3.61	10.19	4.30

IN VITRO	TA1530	G-46	D-3 % CONC	D-3 % SURVIVAL	D X 10E5
TCPD	-	-	1.0	51.2	33
NC	-	-	-	100.0	5
PC	+	+	0.5	68.8	267

STOP

c. HOST-MEDIATED ASSAY DATA SHEETS

CONTRACT FDA 71-268

COMPOUND FDA 71-56

PROPYLENE GLYCOL

Host-Mediated Assay - Adjusted Raw CFU X 10^7/0.6 ml

The true raw colony counts were lost through automation for this compound. Thus, the source of the adjusted raw CFU X 10^7/0.6 ml (Column A) was the true raw counts as assimilated by the automatic colony counter, multiplied by the automatic program by 0.16666666666667 (Column B) and then divided by 0.1667 (the check figure). The original concept was that the true CFU X 10^7/0.6 ml would be printed as column A. Through the programing anomaly the Column B check figure was obtained as the raw CFU X 10^7/0.6 ml and recorded as such.

Step 1: Technician set counter - plates on counter.
Step 2: Automatic equipment accumulates counts on 3 plates of 10^{-6} dilution as CFU X 10^7/0.6 ml.
Step 3: Automatic equipment multiplies count obtained in step 1 by 0.16666666666667 to obtain total count/ml at 10^8.
Step 4: Automatic check of result of step 3. TC X 10^8 ÷ 0.1667 = CFU X 10^7/0.6 ml.
Step 5: Technician was to record the true raw CFU X 10^7/0.6 ml in log book, however, through error the computer provided the Column B check figure as the raw count.

To clarify the problem Column A is headed Adjusted Raw CFU X 10E^7/ 0.6 ml in each case where the check figure was provided as the raw count.

HOST MEDIATED ASSAY REPORT SHEET

TEST COMPOUND: FDA 71-56 ORGANISM: SALMONELLA TA1530

DOSE LEVEL: NEGATIVE CONTROL - SALINE

TREATMENT: IN VIVO, ORAL, ACUTE DATE STARTED: SEPTEMBER 29, 1972

ANIMAL NUMBER	A RAW CFU X 10E7/0.6ML	B TOTAL CFU X 10E6/1.0ML	C TOTAL NO. MUTANTS X 10E0/1.0ML	D MUTATION FRE (C/B) X 10E-8
1	10.60	1.86	2.00	1.11
2	6.20	1.03	2.00	1.94
3	11.20	1.87	1.00	.54
4	17.40	2.90	4.00	1.38
5	37.80	6.30	2.00	.32
6	48.10	8.02	2.00	.25
7	9.90	1.65	3.00	1.82
8	19.60	3.30	2.00	.61

NO. OF ANIMALS EQUALS 8
TOTAL CFU OUT OF RANGE EQUALS 2

	COL. B (X 10E6)	COL. C (X 10E0)	COL. D (X 10E-8)
MEAN	3.36	2.25	.99
RANGE	6.98	3.00	1.69
MAX	8.02	4.00	1.94
MIN	1.03	1.00	.25

NO OUTLIERS

HOST MEDIATED ASSAY REPORT SHEET

COMPOUND: FDA 71-56 ORGANISM: SALMONELLA TA1530

DOSE LEVEL: POSITIVE CONTROL - DMN - 100 MG/KG

TREATMENT: IN VIVO, ORAL, ACUTE DATE STARTED: SEPTEMBER 29, 1972

ANIMAL NUMBER	A RAW CFU X 10E7/0.6ML	B TOTAL CFU X 10E8/1.0ML	C TOTAL NO. MUTANTS X 10E0/1.0ML	D MUTATION FRE (C/B) X 10E-8
1	57.20	9.53	55.00	5.77
2	45.20	7.53	30.00	3.98
3	36.30	6.05	38.00	6.28
4	24.60	4.10	84.00	20.49
5	12.70	2.12	14.00	6.61
6	37.00	6.17	32.00	5.19
7	6.70	1.12	18.00	16.12
8	25.20	4.20	43.00	10.24

NO. OF ANIMALS EQUALS 8
NO. OF CONTAMINATED EQUALS 1
TOTAL CFU OUT OF RANGE EQUALS 1

	COL. B (X 10E8)	COL. C (X 10E0)	COL. D (X 10E-8)
MEAN	5.10	39.25	9.33
RANGE	8.42	70.00	16.51
MAX	9.53	84.00	20.49
MIN	1.12	14.00	3.98

NO OUTLIERS

HOST MEDIATED ASSAY REPORT SHEET

COMPOUND: FDA 71-56

ORGANISM: SALMONELLA TA1530

DOSE LEVEL: LOW - 30 MG/KG

TREATMENT: IN VIVO, ORAL, ACUTE

DATE STARTED: SEPTEMBER 29, 1972

ANIMAL NUMBER	A RAW CFU X 10E7/0.6ML	B TOTAL CFU X 10E8/1.0ML	C TOTAL NO. MUTANTS X 10E0/1.0ML	D MUTATION FRE (C/B) X 10E-8
1	14.20	2.37	3.00	1.27
2	27.70	4.62	2.00	.43
3	13.70	2.28	4.00	1.75
4	19.70	3.28	5.00	1.52
5	16.10	2.68	3.00	1.12
6	21.50	3.58	1.00	.28
7	17.30	2.88	2.00	.69

NO. OF ANIMALS EQUALS 7
TOTAL CFU OUT OF RANGE EQUALS 3

	COL. B (X 10E8)	COL. C (X 10E0)	COL. D (X 10E-8)
MEAN	3.10	2.86	1.01
RANGE	2.33	4.00	1.47
MAX	4.62	5.00	1.75
MIN	2.28	1.00	.28

NO OUTLIERS

HOST MEDIATED ASSAY REPORT SHEET

COMPOUND: FDA 71-56 ORGANISM: SALMONELLA TA1530

DOSE LEVEL: INTERMEDIATE - 2500 MG/KG

TREATMENT: IN VIVO, ORAL, ACUTE DATE STARTED: SEPTEMBER 29, 1972

ANIMAL NUMBER	A RAW CFU X 10E7/0.6ML	B TOTAL CFU X 10E8/1.0ML	C TOTAL NO. MUTANTS X 10E0/1.0ML	D MUTATION FRE (C/B) X 10E-8	
1	19.20	3.20	13.00	4.06	*
2	9.20	1.53	2.00	1.30	
3	59.70	9.95	5.00	.50	
4	9.96	1.66	4.00	2.41	
5	27.30	4.55	3.00	.66	
6	6.40	1.07	1.00	.94	
7	34.10	5.68	3.00	.53	
8	16.00	2.67	5.00	1.87	
9	13.30	2.22	6.00	2.71	

NO. OF ANIMALS EQUALS 9
NO. OF CONTAMINATED EQUALS 1

	COL. B (X 10E8)	COL. C (X 10E0)	COL. D (X 10E-8)
MEAN	3.61	4.67	1.67
RANGE	8.88	12.00	3.56
MAX	9.95	13.00	4.06
MIN	1.07	1.00	.50

* SUMMARY WITH OUTLIERS REMOVED

	COL. B (X 10E8)	COL. C (X 10E0)	COL. D (X 10E-8)
MEAN	3.67	3.63	1.37
RANGE	8.88	5.00	2.20
MAX	9.95	6.00	2.71
MIN	1.07	1.00	.50

HOST MEDIATED ASSAY REPORT SHEET

COMPOUND: FDA 71-56 ORGANISM: SALMONELLA TA1530

DOSE LEVEL: HIGH - 5000 MG/KG

TREATMENT: IN VIVO, ORAL, ACUTE DATE STARTED: SEPTEMBER 29, 1972

ANIMAL NUMBER	A RAW CFU X 10E7/0.6ML	B TOTAL CFU X 10E8/1.0ML	C TOTAL NO. MUTANTS X 10E0/1.0ML	D MUTATION FRE (C/B) X 10E-8	
1	51.90	8.65	4.00	.46	
2	58.80	9.80	6.00	.61	
3	37.10	6.18	9.00	1.46	
4	44.80	7.47	2.00	.27	
5	45.60	7.60	8.00	1.05	
6	11.80	1.97	6.00	3.05	*
7	38.40	6.40	4.00	.62	

NO. OF ANIMALS EQUALS 7
NO. OF CONTAMINATED EQUALS 3

	COL. B (X 10E8)	COL. C (X 10E0)	COL. D (X 10E-8)
MEAN	6.87	5.57	1.08
RANGE	7.83	7.00	2.78
MAX	9.80	9.00	3.05
MIN	1.97	2.00	.27

* SUMMARY WITH OUTLIERS REMOVED

	COL. B (X 10E8)	COL. C (X 10E0)	COL. D (X 10E-8)
MEAN	7.68	5.50	.75
RANGE	3.62	7.00	1.19
MAX	9.80	9.00	1.46
MIN	6.18	2.00	.27

STOP

HOST MEDIATED ASSAY REPORT SHEET

COMPOUND: FDA 71-56 ORGANISM: SALMONELLA TA1530

DOSE LEVEL: LOW - 30 MG/KG

TREATMENT: IN VIVO, ORAL, SUBACUTE DATE STARTED: SEPTEMBER 29, 1972

ANIMAL NUMBER	A RAW CFU X 10E7/0.6ML	B TOTAL CFU X 10E8/1.0ML	C TOTAL NO. MUTANTS X 10E0/1.0ML	D MUTATION FRE (C/B) X 10E-8	
1	55.10	9.18	2.00	.22	
2	33.20	5.53	1.00	.18	
3	7.70	1.28	1.00	.78	
4	8.50	1.42	5.00	3.53	*
5	14.90	2.48	3.00	1.21	
6	37.00	6.17	2.00	.32	
7	7.98	1.33	2.00	1.50	
8	18.30	3.05	4.00	1.31	

NO. OF ANIMALS EQUALS 8
TOTAL CFU OUT OF RANGE EQUALS 2

	COL. B (X 10E8)	COL. C (X 10E0)	COL. D (X 10E-8)
MEAN	3.81	2.50	1.13
RANGE	7.90	4.00	3.35
MAX	9.18	5.00	3.53
MIN	1.28	1.00	.18

* SUMMARY WITH OUTLIERS REMOVED

	COL. B (X 10E8)	COL. C (X 10E0)	COL. D (X 10E-8)
MEAN	4.15	2.14	.79
RANGE	7.90	3.00	1.32
MAX	9.18	4.00	1.50
MIN	1.28	1.00	.18

HOST MEDIATED ASSAY REPORT SHEET

COMPOUND: FDA 71-56 ORGANISM: SALMONELLA TA1530

DOSE LEVEL: INTERMEDIATE - 2500 MG/KG

TREATMENT: IN VIVO, ORAL, SUBACUTE DATE STARTED: SEPTEMBER 29, 1972

ANIMAL NUMBER	A RAW CFU X 10E7/0.6ML	B TOTAL CFU X 10E8/1.0ML	C TOTAL NO. MUTANTS X 10E0/1.0ML	D MUTATION FRE (C/B) X 10E-8	
1	9.50	1.58	1.00	.63	
2	8.30	1.38	1.00	.72	
3	6.30	1.05	2.00	1.90	
4	6.80	1.13	2.00	1.76	
5	6.70	1.12	2.00	1.79	
6	6.00	1.00	5.00	5.00	*
7	7.70	1.28	3.00	2.34	

NO. OF ANIMALS EQUALS 7
TOTAL CFU OUT OF RANGE EQUALS 3

	COL. B (X 10E8)	COL. C (X 10E0)	COL. D (X 10E-8)
MEAN	1.22	2.29	2.02
RANGE	.58	4.00	4.37
MAX	1.58	5.00	5.00
MIN	1.00	1.00	.63

* SUMMARY WITH OUTLIERS REMOVED

	COL. B (X 10E8)	COL. C (X 10E0)	COL. D (X 10E-8)
MEAN	1.26	1.83	1.53
RANGE	.53	2.00	1.71
MAX	1.58	3.00	2.34
MIN	1.05	1.00	.63

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HOST MEDIATED ASSAY REPORT SHEET

COMPOUND: FDA 71-56

ORGANISM: SALMONELLA TA1530

DOSE LEVEL: HIGH - 5000 MG/KG

TREATMENT: IN VIVO, ORAL, SUBACUTE

DATE STARTED: SEPTEMBER 29, 1972

ANIMAL NUMBER	A RAW CFU X 10E7/0.6ML	B TOTAL CFU X 10E8/1.0ML	C TOTAL NO. MUTANTS X 10E0/1.0ML	D MUTATION FRE (C/B) X 10E-8
1	16.70	2.78	7.00	2.51
2	7.20	1.20	2.00	1.67
3	6.60	1.10	1.00	.91
4	13.10	2.18	8.00	3.66
5	12.50	2.08	1.00	.48
6	51.40	8.57	7.00	.82
7	9.70	1.62	5.00	3.09
8	7.00	1.17	4.00	3.43

NO. OF ANIMALS EQUALS 8
TOTAL CFU OUT OF RANGE EQUALS 2

	COL. B (X 10E8)	COL. C (X 10E0)	COL. D (X 10E-8)
MEAN	2.59	4.38	2.07
RANGE	7.47	7.00	3.18
MAX	8.57	8.00	3.66
MIN	1.10	1.00	.48

NO OUTLIERS

STOP

HOST MEDIATED ASSAY REPORT SHEET

COMPOUND: FDA 71-56 ORGANISM: SALMONELLA G-46

DOSE LEVEL: NEGATIVE CONTROL - SALINE

TREATMENT: IN VIVO, ORAL, ACUTE DATE STARTED: SEPTEMBER 15, 1972

ANIMAL NUMBER	A ADJUSTED RAW CFU X 10E7/0.6ML	B TOTAL CFU X 10E8/1.0ML	C TOTAL NO. MUTANTS X 10E0/1.0ML	D MUTATION FRE (C/B) X 10E-8
1	23.94	3.99	3.00	.75
2	30.90	5.15	4.00	.78
3	24.54	4.09	3.00	.73
4	13.14	2.19	2.00	.91
5	17.34	2.89	4.00	1.38
6	25.98	4.33	3.00	.69
7	12.66	2.11	2.00	.95
8	11.70	1.95	3.00	1.54

NO. OF ANIMALS EQUALS 8
NO. OF CONTAMINATED EQUALS 1
TOTAL CFU OUT OF RANGE EQUALS 1

	COL. B (X 10E8)	COL. C (X 10E0)	COL. D (X 10E-8)
MEAN	3.34	3.00	.97
RANGE	3.20	2.00	.85
MAX	5.15	4.00	1.54
MIN	1.95	2.00	.69

NO OUTLIERS

HOST MEDIATED ASSAY REPORT SHEET

COMPOUND: FDA 71-56 ORGANISM: SALMONELLA G-46

DOSE LEVEL: POSITIVE CONTROL - DMN - 100 MG/KG

TREATMENT: IN VIVO, ORAL, ACUTE DATE STARTED: SEPTEMBER 15, 1972

ANIMAL NUMBER	A ADJUSTED RAW CFU X 10E7/0.6ML	B TOTAL CFU X 10E6/1.0ML	C TOTAL NO. MUTANTS X 10E0/1.0ML	D MUTATION FRE (C/B) X 10E-8
1	18.54	3.09	54.00	17.48
2	36.12	6.02	74.00	12.29
3	24.72	4.12	70.00	16.99
4	30.78	5.13	92.00	17.93
5	46.02	7.67	68.00	8.87
6	10.74	1.79	53.00	29.61
7	23.94	3.99	47.00	11.78
8	20.94	3.49	85.00	24.35
9	40.14	6.69	89.00	13.30

NO. OF ANIMALS EQUALS 9
NO. OF CONTAMINATED EQUALS 1

	COL. B (X 10E6)	COL. C (X 10E0)	COL. D (X 10E-8)
MEAN	4.67	70.22	16.96
RANGE	5.88	45.00	20.74
MAX	7.67	92.00	29.61
MIN	1.79	47.00	8.87

NO OUTLIERS

STOP

HOST MEDIATED ASSAY REPORT SHEET

COMPOUND: FDA 71-56 ORGANISM: SALMONELLA G-46

DOSE LEVEL: LOW - 30 MG/KG

TREATMENT: IN VIVO, ORAL, ACUTE DATE STARTED: SEPTEMBER 15, 1972

ANIMAL NUMBER	A ADJUSTED RAW CFU X 10E7/0.6ML	B TOTAL CFU X 10E8/1.0ML	C TOTAL NO. MUTANTS X 10E0/1.0ML	D MUTATION FRE (C/B) X 10E-8
1	43.98	7.33	4.00	.55
2	24.40	4.07	3.00	.74
3	31.98	5.33	4.00	.75
4	51.18	8.53	6.00	.70
5	58.00	9.67	1.00	.10
6	51.54	8.59	5.00	.58
7	58.50	9.75	1.00	.10

NO. OF ANIMALS EQUALS 7
TOTAL CFU OUT OF RANGE EQUALS 1
SAMPLES WITH ZERO MUTANTS EQUAL 2

	COL. B (X 10E8)	COL. C (X 10E0)	COL. D (X 10E-8)
MEAN	7.61	3.43	.50
RANGE	5.68	5.00	.65
MAX	9.75	6.00	.75
MIN	4.07	1.00	.10

NO OUTLIERS

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HOST MEDIATED ASSAY REPORT SHEET

COMPOUND: FDA 71-56 ORGANISM: SALMONELLA G-46

DOSE LEVEL: INTERMEDIATE - 2500 MG/KG

TREATMENT: IN VIVO, ORAL, ACUTE DATE STARTED: SEPTEMBER 15, 1972

ANIMAL NUMBER	A ADJUSTED RAW CFU X 10E7/0.6ML	B TOTAL CFU X 10E8/1.0ML	C TOTAL NO. MUTANTS X 10E0/1.0ML	D MUTATION FRE (C/B) X 10E-8
1	10.50	1.75	3.00	1.71
2	37.08	6.18	7.00	1.13
3	21.30	3.55	5.00	1.41
4	38.94	6.49	6.00	.92
5	27.54	4.59	4.00	.87
6	18.90	3.15	2.00	.63
7	21.12	3.52	5.00	1.42
8	47.94	7.99	5.00	.63

NO. OF ANIMALS EQUALS 8
NO. OF CONTAMINATED EQUALS 2

	COL. B (X 10E8)	COL. C (X 10E0)	COL. D (X 10E-8)
MEAN	4.65	4.63	1.09
RANGE	6.24	5.00	1.09
MAX	7.99	7.00	1.71
MIN	1.75	2.00	.63

NO OUTLIERS

HOST MEDIATED ASSAY REPORT SHEET

COMPOUND: FDA 71-56 ORGANISM: SALMONELLA G-46

DOSE LEVEL: HIGH - 5000 MG/KG

TREATMENT: IN VIVO, ORAL, ACUTE DATE STARTED: SEPTEMBER 15, 1972

ANIMAL NUMBER	A ADJUSTED RAW CFU X 10E7/0.6ML	B TOTAL CFU X 10E8/1.0ML	C TOTAL NO. MUTANTS X 10E0/1.0ML	D MUTATION FRE (C/B) X 10E-8	
1	11.34	1.89	2.00	1.06	
2	24.30	4.05	2.00	.49	
3	15.30	2.55	2.00	.78	
4	21.54	3.59	4.00	1.11	
5	18.54	3.09	1.00	.32	
6	31.02	5.17	2.00	.39	
7	25.50	4.25	2.00	.47	
8	14.10	2.35	2.00	.85	
9	41.10	6.85	5.00	.73	
10	25.14	4.19	11.00	2.63	*

NO. OF ANIMALS EQUALS 10

	COL. B (X 10E8)	COL. C (X 10E0)	COL. D (X 10E-8)
MEAN	3.80	3.30	.88
RANGE	4.96	10.00	2.30
MAX	6.85	11.00	2.63
MIN	1.89	1.00	.32

* SUMMARY WITH OUTLIERS REMOVED

	COL. B (X 10E8)	COL. C (X 10E0)	COL. D (X 10E-8)
MEAN	3.75	2.44	.69
RANGE	4.96	4.00	.79
MAX	6.85	5.00	1.11
MIN	1.89	1.00	.32

TOP

HOST MEDIATED ASSAY REPORT SHEET

COMPOUND: FDA 71-56 ORGANISM: SALMONELLA G-46

DOSE LEVEL: LOW - 30 MG/KG

TREATMENT: IN VIVO, ORAL, SUBACUTE DATE STARTED: SEPTEMBER 15, 1972

ANIMAL NUMBER	A ADJUSTED RAW CFU X 10E7/0.6ML	B TOTAL CFU X 10E6/1.0ML	C TOTAL NO. MUTANTS X 10E0/1.0ML	D MUTATION FRE (C/B) X 10E-8
1	28.80	4.80	4.00	.83
2	25.20	4.20	4.00	.95
3	10.00	1.67	3.00	1.80
4	37.50	6.25	5.00	.80
5	16.50	2.75	5.00	1.82
6	37.00	6.17	5.00	.81
7	19.70	3.28	4.00	1.22
8	32.50	5.42	4.00	.74
9	25.40	4.23	3.00	.71

NO. OF ANIMALS EQUALS 9
TOTAL CFU OUT OF RANGE EQUALS 1

	COL. B (X 10E6)	COL. C (X 10E0)	COL. D (X 10E-8)
MEAN	4.31	4.11	1.08
RANGE	4.58	2.00	1.11
MAX	6.25	5.00	1.82
MIN	1.67	3.00	.71

NO OUTLIERS

STOP

HOST MEDIATED ASSAY REPORT SHEET

COMPOUND: FDA 71-56 ORGANISM: SALMONELLA G-46

DOSE LEVEL: INTERMEDIATE - 2500 MG/KG

TREATMENT: IN VIVO, ORAL, SUBACUTE DATE STARTED: SEPTEMBER 15, 1972

ANIMAL NUMBER	A ADJUSTED RAW CFU X 10E7/0.6ML	B TOTAL CFU X 10E8/1.0ML	C TOTAL NO. MUTANTS X 10E0/1.0ML	D MUTATION FRE (C/B) X 10E-8
1	36.70	6.12	7.00	1.14
2	22.20	3.70	5.00	1.35
3	59.80	9.97	11.00	1.10
4	19.70	3.28	6.00	1.83
5	21.50	3.58	5.00	1.40
6	42.30	7.05	9.00	1.28
7	25.30	4.22	12.00	2.85
8	12.40	2.07	6.00	2.90

NO. OF ANIMALS EQUALS 8
NO. OF DEAD ANIMALS EQUALS 1
TOTAL CFU OUT OF RANGE EQUALS 1

	COL. B (X 10E8)	COL. C (X 10E0)	COL. D (X 10E-8)
MEAN	5.00	7.63	1.73
RANGE	7.90	7.00	1.80
MAX	9.97	12.00	2.90
MIN	2.07	5.00	1.10

NO OUTLIERS

STOP

HOST MEDIATED ASSAY REPORT SHEET

COMPOUND: FDA 71-56 ORGANISM: SALMONELLA G-46

DOSE LEVEL: HIGH - 5000 MG/KG

TREATMENT: IN VIVO, ORAL, SUBACUTE DATE STARTED: SEPTEMBER 15, 1972

ANIMAL NUMBER	A ADJUSTED RAW CFU X 10E7/0.6ML	B TOTAL CFU X 10E8/1.0ML	C TOTAL NO. MUTANTS X 10E0/1.0ML	D MUTATION FRE (C/B) X 10E-8
1	44.90	7.48	17.00	2.27
2	57.80	9.63	24.00	2.49
3	24.30	4.05	14.00	3.46
4	7.00	1.17	8.00	6.86
5	57.80	9.63	12.00	1.25
6	6.42	1.07	7.00	6.54
7	56.50	9.42	9.00	.96
8	11.40	1.90	8.00	4.21

NO. OF ANIMALS EQUALS 8
TOTAL CFU OUT OF RANGE EQUALS 2

	COL. B (X 10E8)	COL. C (X 10E0)	COL. D (X 10E-8)
MEAN	5.54	12.38	3.50
RANGE	8.56	17.00	5.90
MAX	9.63	24.00	6.86
MIN	1.07	7.00	.96

NO OUTLIERS

STOP

HOST MEDIATED ASSAY REPORT SHEET

COMPOUND: FDA 71-56 ORGANISM: SACCHAROMYCES D-3

DOSE LEVEL: NEGATIVE CONTROL - SALINE

TREATMENT: IN VIVO, ORAL, ACUTE DATE STARTED: AUGUST 25, 1972

ANIMAL NUMBER	A ADJUSTED RAW CFU X 10E5/1.0ML	B TOTAL CFU SCREENED X 10E5/1.0ML	C TOTAL RECOMBINANTS /1.0ML	D RECOMB/CFU SCREENED X 10E-5	
1	810.00	.81	2.00	2.47	
2	451.00	.45	0.	0.	
3	814.00	.81	1.00	1.23	
4	740.00	.74	1.00	1.35	
5	236.00	.24	2.00	8.47	*
6	415.00	.42	2.00	4.82	
7	472.00	.47	2.00	4.24	
8	710.00	.71	1.00	1.41	
TOTAL		4.65	11.00		

NO. OF ANIMALS EQUALS 8
TOTAL SCREENED OUT OF RANGE EQUALS 2

MEAN C/MEAN B = 2.37

	COL. B (X 10E5)	COL. C (X 10E0)	COL. D (X 10E-5)
MEAN	.58	1.38	3.00
RANGE	.58	2.00	8.47
MAX	.81	2.00	8.47
MIN	.24	0.	0.

* SUMMARY WITH OUTLIERS REMOVED

MEAN C/MEAN B = 2.04

	COL. B (X 10E5)	COL. C (X 10E0)	COL. D (X 10E-5)
MEAN	.63	1.29	2.22
RANGE	.40	2.00	4.82
MAX	.81	2.00	4.82
MIN	.42	0.	0.

STOP

HOST MEDIATED ASSAY REPORT SHEET

COMPOUND: FDA 71-56 ORGANISM: SACCHAROMYCES D-3

DOSE LEVEL: POSITIVE CONTROL - EMS - 350 MG/KG I.M.

TREATMENT: IN VIVO, ORAL, ACUTE DATE STARTED: AUGUST 25, 1972

ANIMAL NUMBER	A ADJUSTED RAW CFU X 10E5/1.0ML	B TOTAL CFU SCREENED X 10E5/1.0ML	C TOTAL RECOMBINANTS /1.0ML	D RECOMB/CFU SCREENED X 10E-5	
1	713.00	.71	11.00	15.43	
2	844.00	.84	17.00	20.14	
3	242.00	.24	14.00	57.85	*
4	590.00	.59	14.00	23.73	
5	632.00	.63	10.00	15.82	
6	798.00	.80	22.00	27.57	
7	885.00	.88	25.00	28.25	
8	526.00	.53	15.00	28.52	
9	537.00	.54	16.00	29.80	
TOTAL		5.77	144.00		

NO. OF ANIMALS EQUALS 9
NO. OF DEAD ANIMALS EQUALS 1

MEAN C/MEAN B = 24.97

	COL. B (X 10E5)	COL. C (X 10E0)	COL. D (X 10E-5)
MEAN	.64	16.00	27.46
RANGE	.64	15.00	42.42
MAX	.88	25.00	57.85
MIN	.24	10.00	15.43

* SUMMARY WITH OUTLIERS REMOVED

MEAN C/MEAN B = 23.53

	COL. B (X 10E5)	COL. C (X 10E0)	COL. D (X 10E-5)
MEAN	.69	16.25	23.66
RANGE	.36	15.00	14.37
MAX	.88	25.00	29.80
MIN	.53	10.00	15.43

TOP

HOST MEDIATED ASSAY REPORT SHEET

COMPOUND: FDA 71-56 ORGANISM: SACCHAROMYCES D-3

DOSE LEVEL: LOW - 30 MG/KG

TREATMENT: IN VIVO, ORAL, ACUTE DATE STARTED: AUGUST 25, 1972

ANIMAL NUMBER	A ADJUSTED RAW CFU X 10E5/1.0ML	B TOTAL CFU SCREENED X 10E5/1.0ML	C TOTAL RECOMBINANTS /1.0ML	D RECOMB/CFU SCREENED X 10E-5	
1	742.00	.74	11.00	14.82	
2	800.00	.80	12.00	15.00	
3	746.00	.75	14.00	18.77	
4	681.00	.68	21.00	30.84	*
5	286.00	.29	0.	0.	
6	752.00	.75	0.	0.	
7	211.00	.21	1.00	4.74	
8	501.00	.50	0.	0.	
TOTAL		4.72	59.00		

NO. OF ANIMALS EQUALS 8
TOTAL SCREENED OUT OF RANGE EQUALS 2

MEAN C/MEAN B = 12.50

	COL. B (X 10E5)	COL. C (X 10E0)	COL. D (X 10E-5)
MEAN	.59	7.38	10.52
RANGE	.59	21.00	30.84
MAX	.80	21.00	30.84
MIN	.21	0.	0.

* SUMMARY WITH OUTLIERS REMOVED

MEAN C/MEAN B = 9.41

	COL. B (X 10E5)	COL. C (X 10E0)	COL. D (X 10E-5)
MEAN	.55	5.43	7.62
RANGE	.59	14.00	18.77
MAX	.80	14.00	18.77
MIN	.21	0.	0.

STOP

HOST MEDIATED ASSAY REPORT SHEET

COMPOUND: FDA 71-56 ORGANISM: SACCHAROMYCES D-3

DOSE LEVEL: INTERMEDIATE - 2500 MG/KG

TREATMENT: IN VIVO, ORAL, ACUTE DATE STARTED: AUGUST 25, 1972

ANIMAL NUMBER	A ADJUSTED RAW CFU X 10E5/1.0ML	B TOTAL CFU SCREENED X 10E5/1.0ML	C TOTAL RECOMBINANTS /1.0ML	D RECOMB/CFU SCREENED X 10E-5
1	651.00	.65	12.00	18.43
2	750.00	.75	11.00	14.67
3	392.00	.39	14.00	35.71
4	314.00	.31	12.00	38.22
5	371.00	.37	11.00	29.65
6	410.00	.41	13.00	31.71
7	721.00	.72	21.00	29.13
8	451.00	.45	3.00	6.65
TOTAL		4.06	97.00	

NO. OF ANIMALS EQUALS 8
TOTAL SCREENED OUT OF RANGE EQUALS 2

MEAN C/MEAN B = 23.89

	COL. B (X 10E5)	COL. C (X 10E0)	COL. D (X 10E-5)
MEAN	.51	12.13	25.52
RANGE	.44	18.00	31.56
MAX	.75	21.00	38.22
MIN	.31	3.00	6.65

NO OUTLIERS

STOP

HOST MEDIATED ASSAY REPORT SHEET

COMPOUND: FDA 71-56 ORGANISM: SACCHAROMYCES D-4

DOSE LEVEL: HIGH - 5000 MG/KG

TREATMENT: IN VIVO, ORAL, ACUTE DATE STARTED: AUGUST 25, 1972

ANIMAL NUMBER	A ADJUSTED RAW CFU X 10E5/1.0ML	B TOTAL CFU SCREENED X 10E5/1.0ML	C TOTAL RECOMBINANTS /1.0ML	D RECOMB/CFU SCREENED X 10E-5
1	183.00	.18	0.	0.
2	100.00	.10	0.	0.
3	143.00	.14	1.00	6.99
4	214.00	.21	1.00	4.67
5	251.00	.25	2.00	7.97
6	132.00	.13	1.00	7.58
7	532.00	.53	2.00	3.76
8	140.00	.14	0.	0.
TOTAL		1.69	7.00	

NO. OF ANIMALS EQUALS 8
TOTAL SCREENED OUT OF RANGE EQUALS 2

MEAN C/MEAN B = 4.13

	COL. B (X 10E5)	COL. C (X 10E0)	COL. D (X 10E-5)
MEAN	.21	.88	3.87
RANGE	.43	2.00	7.97
MAX	.53	2.00	7.97
MIN	.10	0.	0.

NO OUTLIERS

STOP

HOST MEDIATED ASSAY REPORT SHEET

COMPOUND: FDA 71-56 ORGANISM: SACCHAROMYCES D-3

DOSE LEVEL: LOW - 30 MG/KG

TREATMENT: IN VIVO, ORAL, SUBACUTE DATE STARTED: AUGUST 25, 1972

ANIMAL NUMBER	A ADJUSTED RAW CFU X 10E5/1.0ML	B TOTAL CFU SCREENED X 10E5/1.0ML	C TOTAL RECOMBINANTS /1.0ML	D RECOMB/CFU SCREENED X 10E-5
1	725.00	.72	10.00	13.79
2	633.00	.63	6.00	9.48
3	544.00	.54	10.00	18.38
4	810.00	.81	10.00	12.35
5	700.00	.70	3.00	4.29
6	471.00	.47	4.00	8.49
7	631.00	.63	3.00	4.75
8	428.00	.43	4.00	9.35
TOTAL		4.94	50.00	

NO. OF ANIMALS EQUALS 8
TOTAL SCREENED OUT OF RANGE EQUALS 2

MEAN C/MEAN B = 10.12

	COL. B (X 10E5)	COL. C (X 10E0)	COL. D (X 10E-5)
MEAN	.62	6.25	10.11
RANGE	.38	7.00	14.10
MAX	.81	10.00	18.38
MIN	.43	3.00	4.29

NO OUTLIERS

STOP

HOST MEDIATED ASSAY REPORT SHEET

COMPOUND: FDA 71-56 ORGANISM: SACCHAROMYCES D-3

DOSE LEVEL: INTERMEDIATE - 2500 MG/KG

TREATMENT: IN VIVO, ORAL, SUBACUTE DATE STARTED: AUGUST 25, 1972

ANIMAL NUMBER	A ADJUSTED RAW CFU X 10E5/1.0ML	B TOTAL CFU SCREENED X 10E5/1.0ML	C TOTAL RECOMBINANTS /1.0ML	D RECOMB/CFU SCREENED X 10E-5	
1	440.00	.44	6.00	13.64	*
2	421.00	.42	4.00	9.50	
3	530.00	.53	3.00	5.66	
4	473.00	.47	4.00	8.46	
5	410.00	.41	2.00	4.88	
6	150.00	.15	1.00	6.67	
7	330.00	.33	2.00	6.06	
TOTAL		2.75	22.00		

NO. OF ANIMALS EQUALS 7
TOTAL SCREENED OUT OF RANGE EQUALS 3

MEAN C/MEAN B = 7.99

	COL. B (X 10E5)	COL. C (X 10E0)	COL. D (X 10E-5)
MEAN	.39	3.14	7.84
RANGE	.38	5.00	8.76
MAX	.53	6.00	13.64
MIN	.15	1.00	4.88

* SUMMARY WITH OUTLIERS REMOVED

MEAN C/MEAN B = 6.91

	COL. B (X 10E5)	COL. C (X 10E0)	COL. D (X 10E-5)
MEAN	.39	2.67	6.87
RANGE	.38	3.00	4.62
MAX	.53	4.00	9.50
MIN	.15	1.00	4.88

SUP

3. Cytogenetics

a. In vivo

(1) Acute study

The three compound dosage groups were within the historical range of negative controls with respect to breaks (0-6%). The LD_5 group was somewhat higher than the negative controls in this study but not significantly higher. The 24-hour LD_5 group contained one dicentric chromosome which could be considered as a random event as this type of aberration has been observed in negative control animals. The LD_5 6-hour group exhibited a mitotic index of 4% which was close to the positive control value of 3%. This 4% value was not significantly different from the other treatment groups. In general the treatment groups' mitotic indices were somewhat lower than those of the negative control. The positive control contained 6% cells with severe chromosomal damage (>10 aberrations/cell) together with the other aberrations noted on the summary sheets.

(2) Subacute study

The negative controls contained 2% cells with breaks and one cell had a dicentric chromosome. While this is an infrequent finding it has been observed in negative controls in the past. The compound dosage groups were within the range of the negative controls with respect to breaks.

b. In vitro

The high toxicity of this substance in vitro was evidenced by complete destruction of the cells from the surface of the glass in vitro. The 0.1 mcg/ml level was low enough to permit a low mitotic index. The negative controls and the three compound dosage levels exhibited essentially

normal anaphase chromosomes. The positive control contained five cells with pulverization and fragmentation of the chromosomes within the cell.

c. CYTOGENETICS SUMMARY SHEETS

CONTRACT FDA 71-268

COMPOUND FDA 71-56

PROPYLENE GLYCOL

PROPYLENE GLYCOL
FDA 71-56
ACUTE STUDY
METAPHASE SUMMARY SHEET

Compound	Dosage (mg/kg)	Time*	No. of Animals	No. of Cells	Mitotic Index %	% Cells with Breaks	% Cells with Reunion	% Cells Other Aber.**	% Cells with Aber.
Negative Control	Saline	6	3	150	10	1.4	0	0	1.4
		24	3	150	12	0	0	0	0
		48	3	150	9	0	0	0	0
Low Level	30.0	6	5	250	6	0	0	0	0
		24	5	250	6	3	0	0	3
		48	5	250	6	1	0	0	1
Intermediate Level	2500	6	5	250	7	2	0	0	2
		24	5	250	6	2	0	0	2
		48	5	250	8	0	0	0	0
High Level	5000	6	5	250	4	2	0	0	2
		24	5	250	8	4	1	0	4
		48	5	250	6	6	0	0	6
Positive Control TEM	0.3	48	5	250	3	22	10	6a	36

*Time of kill after injection (hours).
**Cells that have polyploidy (P), pulverization (pp), or greater than 10 aberrations (a).

PROPYLENE GLYCOL
FDA 71-56
SUBACUTE STUDY
METAPHASE SUMMARY SHEET

Compound	Dosage* (mg/kg)	No. of Animals	No. of Cells	Mitotic Index %	% Cells with Breaks	% Cells with Reunion	% Cells Other Aber.**	% Cells with Aber.
Negative Control	Saline	3	150	8	2	0	1	3
Low Level	30.0	5	250	6	2	0	0	2
Intermediate Level	2500	5	250	7	3	0	0	3
High Level	5000	5	250	7	3	0	0	3

*Dosage 1X/day X 5 days.
**Cells that have polyploidy (P), pulverization (pp), or greater than 10 aberrations (a).

PROPYLENE GLYCOL
FDA 71-56
ANAPHASE SUMMARY SHEET

Compound	Dosage (mcg/ml)	Mitotic Index	No. of Cells	% Cells with Acentric Frag.	% Cells with Bridges	% Multipolar Cells	% Cells Other Aber.	% Cells with Aber.
Low Level	0.001	2	100	0	0	0	0	0
Intermediate Level	0.01	2	100	0	0	0	0	0
High Level	0.1	1	100	1	0	0	0	1
Negative Control	Saline	3	100	1	1	0	0	2
Positive Control (TEM)	0.1	1	100	10	7	2	5pp	24

*Cells that polyploidy (P), pulverization (pp), or greater than 10 aberrations (a).

4. Dominant Lethal Assay

The interpretation of these data were made by Dr. David Brusick, Assistant Professor of Microbiology, Howard University, Washington, D. C., as a consultant to Litton Bionetics, Inc.

Fertility Index

Acute - A number of dose levels exhibited increased fertility compared to the negative control value. This was especially true for week 3 where the fertility was unusually low compared to historical fertility values.

Subacute - No notable findings except that the fertility at week 1 was unusually low compared to historical controls.

Average Number of Implantations per Pregnant Female

Acute - Several isolated decreases in average implants per pregnant female were observed. In no cases were the decreases severe and they showed no pattern indicating an induced effect.

Subacute - No indication of any chemically-induced effects.

Average Copora Lutea per Pregnant Female

Acute - The two highest dose levels at weeks 1 and 5 showed significant decreases in numbers of corpora lutea. The negative controls for both of these weeks were unusually high which could account for the decreases. Doses at weeks 4 and 7 also showed significant decreases. Again, both negative controls for these weeks were unusually high.

Subacute - Increased number of corpora lutea at all three doses in week 4. Slight decrease at intermediate dose at week 1.

Average Pre-Implantation Losses per Pregnant Female

Acute - Except for the high dose at week 2, there were no indications of chemically-induced pre-implantation losses of a substantial level.

Subacute - The negative controls appeared high at several weeks. Increases in pre-implantation losses were seen at the low dose levels in weeks 2, 4 and 5. A number of doses showed increases compared to the historical controls.

Average Dead Implantations per Pregnant Female

Acute - Week 3 looked suspicious except that a negative control of 0/8 was not a good comparison for the three dose levels. The low and intermediate doses at week 3 showed substantial numbers of dead implants whereas the number at the high dose dropped considerably.

Subacute - Week 6 showed a significant reverse dose response. This apparent response seemed to be directly related to the extremely low negative control. None of the values differed significantly from the historical controls.

Females with One or More Dead Implants

Acute - These data indicated that the majority of dead implants were seen in a little better than one-half of the females at the intermediate dose and less than one-half of the females at the low and high doses.

Subacute - The same problem appeared at week 6 also. The historical control must be used to evaluate the results in this situation.

Females with Two or More Dead Implants

Acute - No notable findings.

Subacute - No indication of chemically-induced effects.

Dead Implants per Total Implants

Acute - The data appeared to implicate week 3 as possibly showing dominant lethality. The fact that only 98 implants were observed in the females at this week weakened the implication considerably. The low fertility observed in the negative controls might indicate a problem with the control animals. This problem may suggest chemically-induced dominant lethality, but the historical controls would not seem to support such a suggestion.

Subacute - Week 6 appeared to show effect when compared to the negative control but not when compared to historical controls.

FINAL EVALUATION SUMMARY

Compound 56 showed no significant activity in this dominant lethal evaluation and is considered negative. Some apparent activity observed at certain dose levels at several weeks was considered to be a result of suboptimal controls and not to chemically-induced effects.

DOMINANT LETHAL ASSAY

SUMMARY TABLES

CONTRACT FDA 71-268

COMPOUND FDA 71-56

PROPYLENE GLYCOL

TABLE I

COMPOUND 56 STUDY ACUTE

FERTILITY INDEX

LOG DOSE	ARITH DOSE	WEEK	HISTORICAL CONTROL	NEGATIVE CONTROL	DOSE LEVEL 30.000 MG/KG	DOSE LEVEL 2500.000 MG/KG	DOSE LEVEL 5000.000 MG/KG	POSITIVE CONTROL
!	!	1	83/119=0.70	12/20=0.60	11/19=0.58	10/19=0.53	9/20=0.45	10/20=0.50
!	!						*	
		2	92/119=0.78	11/20=0.55	17/20=0.85*	12/20=0.60	15/20=0.75	4/20=0.20*
				*				**
		3	96/118=0.82	8/20=0.40	16/20=0.80**	15/20=0.75*	15/20=0.75*	3/20=0.15
				**				**
		4	104/120=0.87	14/20=0.70	18/20=0.90	16/20=0.80	19/20=0.95*	5/20=0.25**
								**
		5	95/119=0.80	15/20=0.75	17/20=0.85	17/20=0.85	19/20=0.95	11/20=0.55
								*
		6	96/119=0.81	13/20=0.65	18/20=0.90	17/20=0.85	19/20=0.95*	16/20=0.80
		7	103/118=0.88	14/20=0.70	15/20=0.75	20/20=1.00**	18/20=0.90	19/20=0.95*
				*				
		8	102/120=0.85	14/20=0.70	17/20=0.85	17/20=0.85	15/20=0.75	18/20=0.90

SYMBOLS ON FIRST LINE DENOTE SIGNIFICANT RELATIONSHIPS AND DIFFERENCES USING THE NEGATIVE CONTROL GROUP

SYMBOLS ON SECOND LINE DENOTE SIGNIFICANT RELATIONSHIPS AND DIFFERENCES USING THE HISTORICAL CONTROL GROUP

ONE !,* = SIGNIFICANT AT P LESS THAN 0.05
TWO !,* = SIGNIFICANT AT P LESS THAN 0.01

* SIGNIFICANTLY DIFFERENT FROM CONTROL
! SIGNIFICANT LINEAR RELATIONSHIP WITH ARITH OR LOG DOSE (HEADING OF COLUMN)

TABLE II

COMPOUND 56 STUDY ACUTE

AVERAGE NUMBER OF IMPLANTATIONS PER PREGNANT FEMALE

LOG DOSE	ARITH DOSE	WEEK	HISTORICAL CONTROL	NEGATIVE CONTROL	DOSE LEVEL 30.000 MG/KG	DOSE LEVEL 2500.000 MG/KG	DOSE LEVEL 5000.000 MG/KG	POSITIVE CONTROL
! & !		1	1026/ 83=12.4	154/12=12.8	131/11=11.9	100/10=10.0*@D *@D	104/ 9=11.6	102/10=10.2*@D @D
		2	1099/ 92=12.0	124/11=11.3	195/17=11.5	150/12=12.5	186/15=12.4	32/ 4= 8.0
		3	1178/ 96=12.3	98/ 8=12.3	183/16=11.4	174/15=11.6	187/15=12.5	37/ 3=12.3
		4	1231/104=11.8	177/14=12.6	225/18=12.5	193/16=12.1	227/19=12.0	54/ 5=10.8
		5	1121/ 95=11.8	169/15=11.3	212/17=12.5	211/17=12.4	235/19=12.4	129/11=11.7
		6	1125/ 96=11.7	167/13=12.9 *@I	221/18=12.3	179/17=10.5*@@D	238/19=12.5	193/16=12.1
		7	1260/103=12.2	176/14=12.6	166/15=11.1@D	241/20=12.1	226/18=12.6	222/19=11.7
		8	1192/102=11.7	161/14=11.5	191/17=11.2	194/17=11.4	174/15=11.6	205/18=11.4

SYMBOLS ON FIRST LINE DENOTE SIGNIFICANT RELATIONSHIPS AND DIFFERENCES USING THE NEGATIVE CONTROL GROUP

SYMBOLS ON SECOND LINE DENOTE SIGNIFICANT RELATIONSHIPS AND DIFFERENCES USING THE HISTORICAL CONTROL GROUP

& AND * = TWO-TAILED TEST
! AND @ = ONE-TAILED TEST

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*,@ SIGNIFICANTLY DIFFERENT FROM CONTROL
&,! SIGNIFICANT RELATIONSHIP WITH ARITH OR LOG DOSE (HEADING OF COLUMN)

TABLE III

COMPOUND 56 STUDY ACUTE

AVERAGE CORPORA LUTEA PER PREGNANT FEMALE

LOG DOSE	ARITH DOSE	WEEK	HISTORICAL CONTROL	NEGATIVE CONTROL	DOSE LEVEL 30.000 MG/KG	DOSE LEVEL 2500.000 MG/KG	DOSE LEVEL 5000.000 MG/KG	POSITIVE CONTROL
&&!!	& !!	1	1126/ 83=13.6	196/12=16.3 **@@I	152/11=14.7	131/10=13.1**@@D	120/ 9=13.3*@@D	136/10=13.6*@D
&&!! &&!!		2	1220/ 92=13.3	139/11=12.6	234/17=13.8*@I	172/12=14.3*@I	227/15=15.1**@@I *@@I	52/ 4=13.0
!	!	3	1254/ 96=13.1	110/ 8=13.8	208/16=13.0	205/15=13.7	212/15=14.1 @I	40/ 3=13.3
&&!!	& !!	4	1316/104=12.7	216/14=15.4 **@@I	251/18=13.9 @I	217/16=13.6*@D	269/19=14.2 **@@I	65/ 5=13.0*@@D
& ! &&!!	! !	5	1194/ 95=12.6	234/15=15.6 **@@I	251/17=14.8 **@@I	238/17=14.0@D *@I	262/19=13.8*@D @I	140/11=12.7**@@I
&&!!	&&!!	6	1233/ 96=12.8	213/13=16.4 **@@I	286/18=15.9 **@@I	244/17=14.4	289/19=15.2 **@@I	261/16=16.3 **@@I
&&!!	&&!!	7	1319/103=12.8	224/14=16.0 **@@I	208/15=13.9@D	289/20=14.5 **@@I	261/18=14.5 *@I	309/19=16.3 **@@I
		8	1410/102=13.8	189/14=13.5	229/17=13.5	243/17=14.3	204/15=13.6	227/18=12.6

SYMBOLS ON FIRST LINE DENOTE SIGNIFICANT RELATIONSHIPS AND DIFFERENCES USING THE NEGATIVE CONTROL GROUP

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&,! SIGNIFICANT RELATIONSHIP WITH ARITH OR LOG DOSE (HEADING OF COLUMN)

TABLE IV

COMPOUND 56 STUDY ACUTE

AVERAGE PREIMPLANTATION LOSSES PER PREGNANT FEMALE

LOG DOSE	ARITH DOSE	WEEK	HISTORICAL CONTROL	NEGATIVE CONTROL	DOSE LEVEL 30.000 MG/KG	DOSE LEVEL 2500.000 MG/KG	DOSE LEVEL 5000.000 MG/KG	POSITIVE CONTROL
		1	100/ 83= 1.2	42/12= 3.5	31/11= 2.8	31/10= 3.1	16/ 9= 1.8	34/10= 3.4
ε !!				*@I	*@I	*@I		*@@I
		2	121/ 92= 1.3	15/11= 1.4	39/17= 2.3	22/12= 1.8	41/15= 2.7@I	20/ 4= 5.0*@I
εε!! εε!!					@I		**@@I	**@@I
		3	76/ 96= 0.8	12/ 8= 1.5	25/16= 1.6	31/15= 2.1	25/15= 1.7	3/ 3= 1.0
εε!! ε !					@I	*@@I		
		4	85/104= 0.8	39/14= 2.8	26/18= 1.4@D	24/16= 1.5	42/19= 2.2	11/ 5= 2.2
εε!! εε!!				**@@I			**@@I	
εε!! εε!!		5	73/ 95= 0.8	65/15= 4.3	39/17= 2.3@D	27/17= 1.6**@@D	27/19= 1.4**@@D	11/11= 1.0**@@I
εε!!				**@@I	**@@I	@I	@I	
		6	108/ 96= 1.1	46/13= 3.5	65/18= 3.6	65/17= 3.8	51/19= 2.7	68/16= 4.3
εε!! εε!!				**@@I	**@@I	**@@I	**@@I	**@@I
		7	59/103= 0.6	48/14= 3.4	42/15= 2.8	48/20= 2.4	35/18= 1.9	87/19= 4.6
εε!! εε!!				**@@I	**@@I	**@@I	*@@I	**@@I
		8	218/102= 2.1	28/14= 2.0	38/17= 2.2	49/17= 2.9	30/15= 2.0	22/18= 1.2

SYMBOLS ON FIRST LINE DENOTE SIGNIFICANT RELATIONSHIPS AND DIFFERENCES USING THE NEGATIVE CONTROL GROUP

SYMBOLS ON SECOND LINE DENOTE SIGNIFICANT RELATIONSHIPS AND DIFFERENCES USING THE HISTORICAL CONTROL GROUP

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! AND @ = ONE-TAILED TEST

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TWO !,ε,@,* = SIGNIFICANT AT P LESS THAN 0.01

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ε,! SIGNIFICANT RELATIONSHIP WITH ARITH OR LOG DOSE (HEADING OF COLUMN)

TABLE V

COMPOUND 56 STUDY ACUTE

AVERAGE RESORPTIONS (DEAD IMPLANTS) PER PREGNANT FEMALE

LOG DOSE	ARITH DOSE	WEEK	HISTORICAL CONTROL	NEGATIVE CONTROL	DOSE LEVEL 30.000 MG/KG	DOSE LEVEL 2500.000 MG/KG	DOSE LEVEL 5000.000 MG/KG	POSITIVE CONTROL
		1	16/ 83=0.20	4/12=0.34	0/11=0.0 @D **@@D	3/10=0.30	2/ 9=0.23	36/10=3.60**@@I **@@I
		2	35/ 92=0.39	8/11=0.73	5/17=0.30	8/12=0.67	5/15=0.34	3/ 4=0.75
!		3	53/ 96=0.56	0/ 8=0.0 **@@D	13/16=0.82**@@I	17/15=1.14**@@I @I	7/15=0.47**@@I	6/ 3=2.00@I
		4	46/104=0.45	7/14=0.50	7/18=0.39	12/16=0.75	9/19=0.48	13/ 5=2.60
		5	52/ 95=0.55	8/15=0.54	18/17=1.06 @I	9/17=0.53	8/19=0.43	50/11=4.55**@@I **@@I
		6	40/ 96=0.42	5/13=0.39	6/18=0.34	3/17=0.18 @D	8/19=0.43	20/16=1.25*@I *@@I
		7	45/103=0.44	8/14=0.58	12/15=0.80	5/20=0.25	10/18=0.56	14/19=0.74
	!	8	56/102=0.55	9/14=0.65	7/17=0.42	6/17=0.36	25/15=1.67	24/18=1.34 *@@I

SYMBOLS ON FIRST LINE DENOTE SIGNIFICANT RELATIONSHIPS AND DIFFERENCES USING THE NEGATIVE CONTROL GROUP

SYMBOLS ON SECOND LINE DENOTE SIGNIFICANT RELATIONSHIPS AND DIFFERENCES USING THE HISTORICAL CONTROL GROUP

& AND * = TWO-TAILED TEST
! AND @ = ONE-TAILED TEST

ONE !,&,@,* = SIGNIFICANT AT P LESS THAN 0.05
TWO !,&,@,* = SIGNIFICANT AT P LESS THAN 0.01

*,@ SIGNIFICANTLY DIFFERENT FROM CONTROL
&,! SIGNIFICANT RELATIONSHIP WITH ARITH OR LOG DOSE (HEADING OF COLUMN)

TABLE VI

COMPOUND 56 STUDY ACUTE

PROPORTION OF FEMALES WITH ONE OR MORE DEAD IMPLANTATIONS

LOG DOSE	ARITH DOSE	WEEK	HISTORICAL CONTROL	NEGATIVE CONTROL	DOSE LEVEL 30.000 MG/KG	DOSE LEVEL 2500.000 MG/KG	DOSE LEVEL 5000.000 MG/KG	POSITIVE CONTROL
		1	16/ 83=0.20	3/12=0.25	0/11=0.0	3/10=0.30	2/ 9=0.23	7/10=0.70* **
		2	26/ 92=0.29	6/11=0.55	4/17=0.24	5/12=0.42	4/15=0.27	1/ 4=0.25
		3	32/ 96=0.34	0/ 8=0.0 *	7/16=0.44*	9/15=0.60** *	6/15=0.40*	2/ 3=0.67*
		4	34/104=0.33	5/14=0.36	6/18=0.34	6/16=0.38	5/19=0.27	3/ 5=0.60
		5	33/ 95=0.35	3/15=0.20	10/17=0.59*	5/17=0.30	5/19=0.27	11/11=1.00** **
		6	31/ 96=0.33	5/13=0.39	5/18=0.28	2/17=0.12	6/19=0.32	10/16=0.63 *
		7	33/103=0.33	5/14=0.36	6/15=0.40	4/20=0.20	9/18=0.50	9/19=0.48
		8	37/102=0.37	7/14=0.50	5/17=0.30	5/17=0.30	7/15=0.47	13/18=0.73 **

SYMBOLS ON FIRST LINE DENOTE SIGNIFICANT RELATIONSHIPS AND DIFFERENCES USING THE NEGATIVE CONTROL GROUP

SYMBOLS ON SECOND LINE DENOTE SIGNIFICANT RELATIONSHIPS AND DIFFERENCES USING THE HISTORICAL CONTROL GROUP

ONE !,* = SIGNIFICANT AT P LESS THAN 0.05
TWO !,* = SIGNIFICANT AT P LESS THAN 0.01

* SIGNIFICANTLY DIFFERENT FROM CONTROL
! SIGNIFICANT LINEAR RELATIONSHIP WITH ARITH OR LOG DOSE (HEADING OF COLUMN)

TABLE VII

COMPOUND 56 STUDY ACUTE

PORPORTION OF FEMALES WITH TWO OR MORE DEAD IMPLANTATIONS

LOG DOSE	ARITH DOSE	WEEK	HISTORICAL CONTROL	NEGATIVE CONTROL	DOSE LEVEL 30.000 MG/KG	DOSE LEVEL 2500.000 MG/KG	DOSE LEVEL 5000.000 MG/KG	POSITIVE CONTROL
		1	0/ 83=0.0	1/12=0.09 **	0/11=0.0	0/10=0.0	0/ 9=0.0	7/10=0.70** **
		2	9/ 92=0.10	2/11=0.19	1/17=0.06	3/12=0.25	1/15=0.07	1/ 4=0.25
		3	16/ 96=0.17	0/ 8=0.0	2/16=0.13	4/15=0.27	1/15=0.07	2/ 3=0.67* *
		4	9/104=0.09	2/14=0.15	1/18=0.06	4/16=0.25	2/19=0.11	3/ 5=0.60* **
		5	14/ 95=0.15	2/15=0.14	5/17=0.30	2/17=0.12	3/19=0.16	9/11=0.82** **
		6	9/ 96=0.10	0/13=0.0	1/18=0.06	1/17=0.06	1/19=0.06	6/16=0.38* **
		7	8/103=0.08	3/14=0.22	2/15=0.14	1/20=0.05	1/18=0.06	3/19=0.16
		8	16/102=0.16	2/14=0.15	2/17=0.12	1/17=0.06	5/15=0.34	3/18=0.17

SYMBOLS ON FIRST LINE DENOTE SIGNIFICANT RELATIONSHIPS AND DIFFERENCES USING THE NEGATIVE CONTROL GROUP

SYMBOLS ON SECOND LINE DENOTE SIGNIFICANT RELATIONSHIPS AND DIFFERENCES USING THE HISTORICAL CONTROL GROUP

ONE !,* = SIGNIFICANT AT P LESS THAN 0.05
TWO !,* = SIGNIFICANT AT P LESS THAN 0.01

* SIGNIFICANTLY DIFFERENT FROM CONTROL
! SIGNIFICANT LINEAR RELATIONSHIP WITH ARITH OR LOG DOSE (HEADING OF COLUMN)

TABLE VIII

COMPOUND 56 STUDY ACUTE

DEAD IMPLANTS / TOTAL IMPLANTS

WEEK	HISTORICAL CONTROL	NEGATIVE CONTROL	DOSE LEVEL 30.000 MG/KG	DOSE LEVEL 2500.000 MG/KG	DOSE LEVEL 5000.000 MG/KG	POSITIVE CONTROL
1	16/1026=0.02	4/154=0.03	0/131=0.0	3/100=0.03	2/104=0.02	36/102=0.36**@ **@@
2	35/1099=0.04	8/124=0.07	5/195=0.03	8/150=0.06	5/186=0.03	3/ 32=0.10
3	53/1178=0.05	0/ 98=0.0 **@@D	13/183=0.08*@I	17/174=0.10**@@I	7/187=0.04*@I	6/ 37=0.17
4	46/1231=0.04	7/177=0.04	7/225=0.04	12/193=0.07	9/227=0.04	13/ 54=0.25
5	52/1121=0.05	8/169=0.05	18/212=0.09	9/211=0.05	8/235=0.04	50/129=0.39*@I **@@
6	40/1125=0.04	5/167=0.03	6/221=0.03	3/179=0.02	8/238=0.04	20/193=0.11*@I @I
7	45/1260=0.04	8/176=0.05	12/166=0.08	5/241=0.03	10/226=0.05	14/222=0.07
8	56/1192=0.05	9/161=0.06	7/191=0.04	6/194=0.04	25/174=0.15	24/205=0.12 *@I

SYMBOLS ON FIRST LINE DENOTE SIGNIFICANT DIFFERENCES USING
THE NEGATIVE CONTROL GROUP

SYMBOLS ON SECOND LINE DENOTE SIGNIFICANT DIFFERENCES USING
THE HISTORICAL CONTROL GROUP

* = TWO-TAILED TEST
@ = ONE-TAILED TEST

ONE *,@ = SIGNIFICANT AT P LESS THAN 0.05
TWO *,@ = SIGNIFICANT AT P LESS THAN 0.01

*,@ SIGNIFICANTLY DIFFERENT FROM CONTROL

TABLE I

COMPOUND 56 STUDY SUBACUTE

FERTILITY INDEX

LOG DOSE	ARITH DOSE	WEEK	HISTORICAL CONTROL	NEGATIVE CONTROL	DOSE LEVEL 30.000 MG/KG	DOSE LEVEL 2500.000 MG/KG	DOSE LEVEL 5000.000 MG/KG
!!		1	82/119=0.69	10/20=0.50	7/20=0.35 **	10/20=0.50	8/20=0.40 *
!!							
		2	89/120=0.75	15/20=0.75	16/20=0.80	16/20=0.80	13/20=0.65
		3	89/119=0.75	12/20=0.60	15/20=0.75	16/20=0.80	15/20=0.75
		4	91/114=0.80	13/20=0.65	18/20=0.90	19/20=0.95*	15/20=0.75
		5	92/119=0.78	16/20=0.80	16/20=0.80	15/20=0.75	18/20=0.90
		6	101/119=0.85	19/20=0.95	18/20=0.90	16/20=0.80	17/20=0.85
		7	100/115=0.87	17/20=0.85	18/20=0.90	19/20=0.95	17/20=0.85

SYMBOLS ON FIRST LINE DENOTE SIGNIFICANT RELATIONSHIPS AND DIFFERENCES USING THE NEGATIVE CONTROL GROUP

SYMBOLS ON SECOND LINE DENOTE SIGNIFICANT RELATIONSHIPS AND DIFFERENCES USING THE HISTORICAL CONTROL GROUP

ONE !,* = SIGNIFICANT AT P LESS THAN 0.05
TWO !,* = SIGNIFICANT AT P LESS THAN 0.01

* SIGNIFICANTLY DIFFERENT FROM CONTROL
! SIGNIFICANT LINEAR RELATIONSHIP WITH ARITH OR LOG DOSE (HEADING OF COLUMN)

TABLE II

COMPOUND 56 STUDY SUBACUTE

AVERAGE NUMBER OF IMPLANTATIONS PER PREGNANT FEMALE

LOG DOSE	ARITH DOSE	WEEK	HISTORICAL CONTROL	NEGATIVE CONTROL	DOSE LEVEL 30.000 MG/KG	DOSE LEVEL 2500.000 MG/KG	DOSE LEVEL 5000.000 MG/KG
		1	966/ 82=11.8	118/10=11.8	87/ 7=12.4	104/10=10.4	91/ 8=11.4
		2	1115/ 89=12.5	186/15=12.4	175/16=10.9 @D	208/16=13.0	155/13=11.9
		3	1049/ 89=11.8	147/12=12.3	178/15=11.9	192/16=12.0	173/15=11.5
&&!! & !	&&!! &&!!	4	1085/ 91=11.9	136/13=10.5 *@D	191/18=10.6	260/19=13.7**@@I **@@I	196/15=13.1**@@I @I
		5	1110/ 92=12.1	189/16=11.8	171/16=10.7 *@D	194/15=12.9	220/18=12.2
		6	1191/101=11.8	246/19=13.0	212/18=11.8	194/16=12.1	203/17=11.9
		7	1138/100=11.4	214/17=12.6 @I	208/18=11.6	230/19=12.1	190/17=11.2

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&,! SIGNIFICANT RELATIONSHIP WITH ARITH OR LOG DOSE (HEADING OF COLUMN)

TABLE III

COMPOUND 56 STUDY SUBACUTE

AVERAGE CORPORA LUTEA PER PREGNANT FEMALE

LOG DOSE	ARITH DOSE	WEEK	HISTORICAL CONTROL	NEGATIVE CONTROL	DOSE LEVEL 30.000 MG/KG	DOSE LEVEL 2500.000 MG/KG	DOSE LEVEL 5000.000 MG/KG
		1	1079/ 82=13.2	139/10=13.9	98/ 7=14.0	117/10=11.7*@D *@D	106/ 8=13.3
		2	1189/ 89=13.4	206/15=13.7	227/16=14.2	231/16=14.4 @I	184/13=14.2
		3	1125/ 89=12.6	165/12=13.8	195/15=13.0	222/16=13.9	188/15=12.5
& !		4	1134/ 91=12.5	151/13=11.6	277/18=15.4**@@I	283/19=14.9**@@I	207/15=13.8**@@I
&& !	& !			@D	**@@I	**@@I	*@I
		5	1157/ 92=12.6	209/16=13.1	215/16=13.4	200/15=13.3	250/18=13.9
&& !	& !!						*@@I
		6	1268/101=12.6	312/19=16.4	272/18=15.1	252/16=15.8	267/17=15.7
&& !	&& !			**@@I	**@@I	**@@I	**@@I
		7	1215/100=12.2	259/17=15.2	284/18=15.8	285/19=15.0	252/17=14.8
&& !	&& !			**@@I	**@@I	**@@I	**@@I

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&,! SIGNIFICANT RELATIONSHIP WITH ARITH OR LOG DOSE (HEADING OF COLUMN)

TABLE IV

COMPOUND 56 STUDY SUBACUTE

AVERAGE PREIMPLANTATION LOSSES PER PREGNANT FEMALE

LOG DOSE	ARITH DOSE	WEEK	HISTORICAL CONTROL	NEGATIVE CONTROL	DOSE LEVEL 30.000 MG/KG	DOSE LEVEL 2500.000 MG/KG	DOSE LEVEL 5000.000 MG/KG
		1	113/ 82= 1.4	21/10= 2.1	11/ 7= 1.6	13/10= 1.3	15/ 8= 1.9
&&!!	!	2	74/ 89= 0.8	20/15= 1.3	52/16= 3.3@I **@@I	23/16= 1.4 *@@I	29/13= 2.2 @I
& !!		3	76/ 89= 0.9	18/12= 1.5 *@I	17/15= 1.1	30/16= 1.9 **@@I	15/15= 1.0
&&!!	! &&!!	4	49/ 91= 0.5	15/13= 1.2	86/18= 4.8**@@I **@@I	23/19= 1.2 **@@I	11/15= 0.7
		5	47/ 92= 0.5	20/16= 1.3 *@@I	44/16= 2.8@I **@@I	6/15= 0.4*@D	30/18= 1.7
&&!!	&&!!	6	77/101= 0.8	66/19= 3.5 **@@I	60/18= 3.3 **@@I	58/16= 3.6 **@@I	64/17= 3.8 **@@I
&&!!	&&!!	7	77/100= 0.8	45/17= 2.7 *@I	76/18= 4.2 **@@I	55/19= 2.9 **@@I	62/17= 3.7 **@@I

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&,! SIGNIFICANT RELATIONSHIP WITH ARITH OR LOG DOSE (HEADING OF COLUMN)

TABLE V

COMPOUND 56 STUDY SUBACUTE

AVERAGE RESORPTIONS (DEAD IMPLANTS) PER PREGNANT FEMALE

LOG DOSE	ARITH DOSE	WEEK	HISTORICAL CONTROL	NEGATIVE CONTROL	DOSE LEVEL 30.000 MG/KG	DOSE LEVEL 2500.000 MG/KG	DOSE LEVEL 5000.000 MG/KG
		1	33/ 82=0.41	2/10=0.20	1/ 7=0.15	1/10=0.10 *@D	1/ 8=0.13 @D
		2	45/ 89=0.51	4/15=0.27	2/16=0.13 *@@D	6/16=0.38	5/13=0.47
		3	47/ 89=0.53	8/12=0.67	6/15=0.40	10/16=0.63	11/15=0.74
		4	51/ 91=0.57	10/13=0.77	8/18=0.45	16/19=0.85	7/15=0.47
& !! !		5	56/ 92=0.61	15/16=0.94	9/16=0.57	3/15=0.20**@@D *@@D	8/18=0.45@D
		6	46/101=0.46	1/19=0.06 **@@D	10/18=0.56**@@I	7/16=0.44**@@I	6/17=0.36
		7	52/100=0.52	7/17=0.42	18/18=1.00	11/19=0.58	6/17=0.36

SYMBOLS ON FIRST LINE DENOTE SIGNIFICANT RELATIONSHIPS AND DIFFERENCES USING THE NEGATIVE CONTROL GROUP

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TWO !,&,@,* = SIGNIFICANT AT P LESS THAN 0.01

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&,! SIGNIFICANT RELATIONSHIP WITH ARITH OR LOG DOSE (HEADING OF COLUMN)

TABLE VI

COMPOUND 56 STUDY SUBACUTE

PROPORTION OF FEMALES WITH ONE OR MORE DEAD IMPLANTATIONS

LOG DOSE	ARITH DOSE	WEEK	HISTORICAL CONTROL	NEGATIVE CONTROL	DOSE LEVEL 30.000 MG/KG	DOSE LEVEL 2500.000 MG/KG	DOSE LEVEL 5000.000 MG/KG
! !		1	27/ 82=0.33	1/10=0.10	1/ 7=0.15	1/10=0.10	1/ 8=0.13
		2	29/ 89=0.33	3/15=0.20	2/16=0.13	5/16=0.32	5/13=0.39
		3	30/ 89=0.34	4/12=0.34	5/15=0.34	6/16=0.38	6/15=0.40
		4	30/ 91=0.33	8/13=0.62 *	7/18=0.39	9/19=0.48	6/15=0.40
		5	39/ 92=0.43	10/16=0.63	5/16=0.32	3/15=0.20*	5/18=0.28*
		6	32/101=0.32	1/19=0.06 *	8/18=0.45**	7/16=0.44**	4/17=0.24
		7	28/100=0.28	6/17=0.36	9/18=0.50	6/19=0.32	3/17=0.18

SYMBOLS ON FIRST LINE DENOTE SIGNIFICANT RELATIONSHIPS AND DIFFERENCES USING THE NEGATIVE CONTROL GROUP

SYMBOLS ON SECOND LINE DENOTE SIGNIFICANT RELATIONSHIPS AND DIFFERENCES USING THE HISTORICAL CONTROL GROUP

ONE !,* = SIGNIFICANT AT P LESS THAN 0.05
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* SIGNIFICANTLY DIFFERENT FROM CONTROL
! SIGNIFICANT LINEAR RELATIONSHIP WITH ARITH OR LOG DOSE (HEADING OF COLUMN)

TABLE VII

COMPOUND 56 STUDY SUBACUTE

PORPORTION OF FEMALES WITH TWO OR MORE DEAD IMPLANTATIONS

LOG DOSE	ARITH DOSE	WEEK	HISTORICAL CONTROL	NEGATIVE CONTROL	DOSE LEVEL 30.000 MG/KG	DOSE LEVEL 2500.000 MG/KG	DOSE LEVEL 5000.000 MG/KG
		1	5/ 82=0.07	1/10=0.10	0/ 7=0.0	0/10=0.0	0/ 8=0.0
		2	7/ 89=0.08	1/15=0.07	0/16=0.0	1/16=0.07	1/13=0.08
		3	10/ 89=0.12	4/12=0.34 *	1/15=0.07	4/16=0.25	2/15=0.14
		4	12/ 91=0.14	2/13=0.16	1/18=0.06	5/19=0.27	1/15=0.07
		5	14/ 92=0.16	4/16=0.25	4/16=0.25	0/15=0.0 *	3/18=0.17
		6	9/101=0.09	0/19=0.0	1/18=0.06	0/16=0.0	2/17=0.12
		7	13/100=0.13	1/17=0.06	5/18=0.28	3/19=0.16	2/17=0.12

SYMBOLS ON FIRST LINE DENOTE SIGNIFICANT RELATIONSHIPS AND DIFFERENCES USING THE NEGATIVE CONTROL GROUP

SYMBOLS ON SECOND LINE DENOTE SIGNIFICANT RELATIONSHIPS AND DIFFERENCES USING THE HISTORICAL CONTROL GROUP

ONE !,* = SIGNIFICANT AT P LESS THAN 0.05
TWO !,* = SIGNIFICANT AT P LESS THAN 0.01

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! SIGNIFICANT LINEAR RELATIONSHIP WITH ARITH OR LOG DOSE (HEADING OF COLUMN)

TABLE VIII

COMPOUND 56 STUDY SUBACUTE

DEAD IMPLANTS / TOTAL IMPLANTS

WEEK	HISTORICAL CONTROL	NEGATIVE CONTROL	DOSE LEVEL 30.000 MG/KG	DOSE LEVEL 2500.000 MG/KG	DOSE LEVEL 5000.000 MG/KG
1	33/ 966=0.04	2/118=0.02	1/ 87=0.02 @D	1/104=0.01 @D	1/ 91=0.02 @D
2	45/1115=0.05	4/186=0.03	2/175=0.02	6/208=0.03	6/155=0.04
3	47/1049=0.05	8/147=0.06	6/178=0.04	10/192=0.06	11/173=0.07
4	51/1085=0.05	10/136=0.08	8/191=0.05	16/260=0.07	7/196=0.04@D
5	56/1110=0.06	15/189=0.08	9/171=0.06	3/194=0.02**@@D **@@D	8/220=0.04@D
6	46/1191=0.04	1/246=0.01 **@@D	10/212=0.05*@@I	7/194=0.04@I	5/203=0.03
7	52/1138=0.05	7/214=0.04	18/208=0.09*@I	11/230=0.05	6/190=0.04

SYMBOLS ON FIRST LINE DENOTE SIGNIFICANT DIFFERENCES USING
THE NEGATIVE CONTROL GROUP

SYMBOLS ON SECOND LINE DENOTE SIGNIFICANT DIFFERENCES USING
THE HISTORICAL CONTROL GROUP

* = TWO-TAILED TEST
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ONE *,@ = SIGNIFICANT AT P LESS THAN 0.05
TWO *,@ = SIGNIFICANT AT P LESS THAN 0.01

*,@ SIGNIFICANTLY DIFFERENT FROM CONTROL

II. MATERIALS AND METHODS

A. Animal Husbandry

1. Animals (Rats and Mice)

Ten to twelve week old rats (280 to 350 g) and male mice (25 to 30 g) were fed a commerical 4% fat diet and water ad libitum until they were put on experiment. Flow Laboratories random-bred, closed colony, Sprague-Dawley CD strain rats were used in the cytogenetic studies. Flow Laboratories ICR male mice were employed in the Host-Mediated Assay.

2. Preparation of Diet

A commercial 4% fat diet was fed to all animals. Periodic tests to verify the absence of coliforms, Salmonella and Pseudomonas sp. were performed.

3. Husbandry

Animals were held in quarantine for 4-11 days. Mice were housed five to a cage and rats one to five to a cage. Animals were identified by ear punch. Sanitary cages and bedding were used, and changed two times per week, at which time water containers were cleaned, sanitized and filled. Once a week, cages were repositioned on racks; racks were repositioned within rooms monthly. Personnel handling animals or working within animal facilities wore head coverings and face masks, as well as suitable garments. Individuals with respiratory or other overt infections were excluded from the animal facilities.

B. Dosage Determination

1. Acute LD_{50} and LD_5 Determination

Since the compounds proposed for testing are included in

the food additive regulations as "generally recognized as safe" (GRAS), it was expected that a large number of them would be sufficiently non-toxic so that determination of a LD_{50} or a LD_5 would be of no practical value. In fact, this has been our experience with previously tested compounds from this list. In the case of these relatively non-toxic compounds, attempts were made to assure that the amounts to be administered would not affect the animals by means (mechanical, physical, etc.) related to their bulk rather than to their toxicity. In the cases of certain compounds where a LD_{50} or a LD_5 could not be determined, an exceedingly high concentration, 5 g/kg, was employed and accepted as the LD_5 level. In cases where the toxicity was high enough to allow determination of a LD_5, the following protocol was used.

Thirty rats of the strain chosen for studies described below and of approximately the age and weight specified were assigned at random to six groups. Each group was then given, using the chosen route of administration, one of a series of dosages of the test compound following a logarithmic dosage scheme. The series of dosages were derived from a consideration of whatever toxicity information was available for the particular test compound. The objective in selecting dosages was to choose values which would cause mortalities between 10% and 90%.

When information was inadequate to derive a suitable series of dosages, five rats were used to identify the proper range. Each of these was given one of a widely spaced (differing by 10X) series of doses. This was confidently expected to suffice for derivation of the series of dosages to be used in the LD_{50} determination.

The mortalities observed when the series of dosages were given to the 30 rats were then subjected to a probit analysis and calculation of LD_{50}, LD_5, slope and confidence limits by the method of Litchfield and Wilcoxon. The highest dose level used was either a finite LD_5 or 5000 mg/kg. The intermediate level used was either 1/10 of the finite LD_5 or 2500 mg/kg. The low level used was either 1/100 of the finite LD_5 or 30 mg/kg.

2. Subacute Studies

Subacute doses were identical to those used in the acute studies. Each subacute study animal was given the acute dosage once a day for each of five consecutive days (24 hours apart).

C. Mutagenicity Testing Protocols

1. Host-Mediated Assay

Flow Laboratories ICR random-bred male mice were used in this study. In the acute and subacute studies ten animals, 25-30 g each, were employed at each dose level. Solvent and positive controls were run at all times. The positive control (dimethyl nitrosamine) was run by the acute system only at a dose of 100 mg/kg for Salmonella. For yeast, ethyl methane sulfonate (EMS) intramuscularly injected at a dose of 350 mg/kg was used. The solvents used and the toxicity data are presented in the Results and Discussion Section of the report.

The indicator organisms used in this study were: (1) two histidine auxotrophs (his G-46, TA-1530) of Salmonella typhimurium, and (2) a diploid strain (D-3) of Saccharomyces cerevisiae. The induction of reverse mutation was determined with the Salmonella; mitotic recombination was determined with yeast. Chemicals were evaluated directly by in vitro bacterial and yeast studies prior to, or concurrent with, the studies in

mice. Only animals on the subacute studies were not fed the evening prior to compound administration. The Salmonella were carried in tryptone yeast extract gel, transferred weekly. They were transferred to tryptone yeast extract broth 48 hours before use: they were transferred a second time from broth to broth 24 hours prior to use, and again 8 hours before use. The mouse inoculum was prepared by transferring 4 ml of the 8-hour broth culture to 50 ml broth bottles which had been prewarmed at 37°C. Exponential log-phase organisms were inoculated intraperitoneally into the mice approximately 2-1/2 hours later when the appropriate density indicating 3.0×10^8 cells/ml was reached. The Saccharomyces was carried in yeast complete agar. The inoculum was prepared by harvesting the organisms from the surface of the plates with sterile saline. The cells were washed three times with sterile saline and suspended in a concentration of 5.0×10^8 cells/ml. Two ml of the suspension was inoculated into each mouse intraperitoneally. Total plate counts on Salmonella were on tryptone yeast extract and for Saccharomyces on yeast complete medium.

a. Acute study

Three dosage levels (usage, intermediate [determined as discussed previously], and LD_5) were administered orally by intubation to ten mice. Positive controls and negative vehicle controls were included in each study. All animals received 2 ml of the indicator organism intraperitoneally. Each ml contained 3.0×10^8 cells for Salmonella and 5.0×10^8 cells for Saccharomyces. Three hours later, each animal was killed and 2 ml of sterile saline was introduced intraperitoneally. As much fluid as possible was then aseptically removed from the peritoneal cavity. Dilution blanks for bacteria containing 4.5 ml of serile saline were prepared in advance. Tenfold serial

dilutions were made of each peritoneal exudate (0.5 ml exudate + 4.5 ml saline) yielding a concentration series from 10^0 (undiluted peritoneal exudate) through 10^{-7}. For enumeration of total bacterial counts, the 10^{-6} and 10^{-7} dilutions were plated on tryptone yeast extract agar, 3 plates/sample, 0.2 ml sample/plate. Each sample was spread over the surface of the plate using a bent glass rod immersed in 95% ethanol and flamed just prior to use. In plating for the total mutant counts on minimal agar, the 10^0 dilution was used, 0.2 ml being plated on each of 5 plates. The plating procedure was identical to that followed for the tryptone yeast extract agar plates. All plates were incubated at 37°C, tryptone yeast extract agar plates for 18 hours and minimal agar plates for 40 hours. For yeast mitotic recombination, dilution blanks containing 4.5 ml of sterile saline were prepared in advance. Tenfold serial dilutions were made of each sample yielding a series from 10^0 to 10^{-5}. Samples of 0.1 ml of the 10^{-5}, 10^{-4}, and 10^{-3} dilutions were removed and plated on complete medium (10 plates each). All plates were incubated at 30°C for 40 hours. The 10^{-5} dilutions were used to determine total populations and the 10^{-4} and 10^{-3} plates were examined after an additional 40 hours at 4°C for red sectors indicating a mutation. Bacterial scoring was calculated as follows:

Total mutants on 5 plates x appropriate exponent = CFU/ml (CFU is Colony Forming Units) of sample plated CFU/ml x one/dilution factor (10^0 - 10^{-7}) = CFU/ml in undiluted exudate. The mutation frequency (MF) calculated for each sample was:

$$MF = \frac{\text{total mutant cells}}{\text{total population}}$$

$$MFt/MFc = \frac{\text{MF of experimental sample}}{\text{MF of control sample}}$$

(MFt/MFc = 1.00 for control sample)

Yeast mitotic recombinants (presumptive ade 2, his 8 homozygotes) were seen as red colonies or as red sectors on a normally white yeast colony. The plates (from 10^{-4} and 10^{-3} dilutions) were scanned under the 10X lens of a dissecting scope to enumerate the red colonies and sectors. Population determinations were made from the 10^{-5} dilution plates. A recombinant frequency (RF) was calculated:

$$RF = \frac{\text{total recombinants counted}}{\text{total number colonies screened}}$$

b. Subacute study

Similar groups of animals at each dose level received five oral doses of the test compound 24 hours apart. Within 30 minutes after the last dosing, the animals were inoculated with the test organism and handled in the same fashion as those in the acute study.

c. In vitro study

Cultures of S. typhimurium histidine auxotrophs (G-46 and TA-1530) were plated on appropriate media. The test compound was then added to the plate, either in the form of a microdrop of solution (0.01 to 0.25 ml) applied to a small filter paper disc resting on the agar or a small crystal applied directly to the agar. Tenfold serial dilutions of the culture were employed and plated so as not to miss the optimum cell density for mutant growth. Mutant colonies were observed and scored. Strain D-3 Saccharomyces cells at proper dilutions were shaken with the test compound, diluted, and plated at 50% survival level or above (see HMA Supplementary Materials and Methods). Red sectors were then scored and the frequency calculated after suitable incubation. Negative and positive controls were run concurrently. The positive control was EMS for Salmonella and Saccharomyces. The in vitro Salmonella tests were reported

as (+) or (-) or questionable; the in vitro Saccharomyces tests were reported as sample concentrations, percent survival, and recombinants/10^5 survivors. For the Saccharomyces a 50% survival level, e.g., an arbitrary 5.0% w/v test level, was used when no LD_{50} was determinable.

2. Cytogenetic Studies

a. In vivo study

Ten to twelve week old, male, albino rats obtained from a closed colony (random-bred) were used. A total of 59 animals in the acute study and 18 animals in the subacute study was used, as illustrated in the following protocol.

Number of Animals Used

Acute Study

Treatment	Time Killed After Administration		
	6 Hours	24 Hours	48 Hours
High Level	5	5	5
Intermediate Level	5	5	5
Low Level	5	5	5
Positive Control	0	0	5
Negative Control	3	3	3

Subacute Study

Five doses 24 hours apart; animals killed 6 hours after last dose.

Treatment	Killed After Administration
High Level	5
Intermediate Level	5
Low Level	5
Negative Control	3

All animals were dosed by gastric intubation.

Four hours after the last compound administration, and two hours prior to killing, each animal was given 4 mg/kg of colcemid intra-

peritoneally in order to arrest the bone marrow cells in C-mitosis. Animals were killed by using CO_2, and the adhering muscle and epiphysis of one femur were removed. The marrow "plug" was removed with a tuberculin syringe and an 18 gauge needle, aspirated into 5 ml of Hanks' balanced salt solution (BSS) in a test tube and capped. The specimens were centrifuged at 1,500 RPM in a table-top centrifuge for 5 minutes, decanted, and 2 ml of hypotonic 0.5% KCl solution was added with gentle agitation to resuspended the cells. The specimens were then placed in a 37°C water bath for 20 minutes in order to swell the cells. Following centrifugation for 5 minutes at 1,500 RPM, the supernatant was decanted and 2 ml of fixative (3:1 absolute methanol:glacial acetic acid) was added. The cells were resuspended in the fixative with gentle agitation, capped, and placed at 4°C for 30 minutes. The specimens were again centrifuged, decanted, 2 ml of prepared fixative was added, and the cells were resuspended and placed at 4°C overnight.

The following day the specimens were again centrifuged, decanted and 0.3 - 0.6 ml of freshly prepared fixative was added to obtain a suitable density. The cells were resuspended and 2 - 3 drops of the suspension were allowed to drop onto a clean, dry slide held at 15° from the horizontal. As the suspension flowed to the edge of the slide, it was ignited by an alcohol burner and allowed to flame. Following ignition, the slides were allowed to dry at room temperature overnight. Duplicate slides were prepared. The slides were stained using a 5% Giemsa solution (Giemsa buffer pH 7.2) for 20 minutes, rinsed in acetone, 1:1 acetone:xylene, and placed in fresh xylene for 30 minutes. The slides were then mounted using Permount (Fisher Scientific) and 24 x 50 mm coverglasses. The coverglasses were selected to be 0.17 mm $\pm$ 0.005 mm in thickness by use of a coverglass micrometer. The preparations

were examined using Leitz Ortholux I & II microscopes with brightfield optics and xenon light sources. These specimens were scanned with 10X and 24X objectives and suitable metaphase spreads that were countable were then examined critically using 40X, 63X or 100X oil immersion flatfield apochromatic objectives. Oculars were either 12X or 16X widefield periplanatics and the tube magnification either 1X or 1.25X. The filters used were either a didymium (BG20) or a Schott IL570 mμ interference filter.

The chromosomes of each cell were counted and only diploid cells were analyzed. They were scored for chromatid gaps and breaks, chromosome gaps and breaks, reunions, cells with greater than ten aberrations, polyploidy, pulverization, and any other chromosomal aberrations which were observed. They were recorded on the currently used forms and expressed as percentages on the summary sheets. Fifty metaphase spreads were scored per animal. Mitotic indices were obtained by counting at least 500 cells and the ratio of the number of cells in mitosis/the number of cells observed was expressed as the mitotic index.

Positive controls in the acute study consisted of animals which had been given the known mutagen Triethylene Melamine (TEM) administered intraperitoneally at a level of 0.30 mg/kg. Negative controls on the acute and subacute studies consisted of the vehicle in which the compound was administered. The dosage levels, solvents and toxicity data are included in the Results and Discussion Section of the report.

b. In vitro study

Human embryonic lung cultures (WI-38) which were negative for adventitious agents (viruses, mycoplasma) which may interfere

were used. These cells were employed at passage level 19. The cells had been transferred using 0.025% trypsin and planted in 32 oz. prescription bottles containing 40 ml of tissue culture medium. When growth was approximately 95% confluent the cells were removed from the glass using trypsin, centrifuged, and frozen in tissue culture medium containing dimethyl sulfoxide (DMSO). Cells were frozen in vials in the vapor phase of liquid nitrogen at a concentration of 2×10^6 cells/ml. When needed, the vials were removed from liquid nitrogen, quick-thawed in a 37°C water bath, washed free of DMSO, suspended in tissue culture medium (minimal essential medium [MEM] plus 1% glutamine, 200 units/ml of penicillin and 200 µg/ml of streptomycin and 15% fetal calf serum) and planted in milk dilution bottles at a concentration of 5×10^5 cells/ml. The test compound was added at three dose levels using three bottles for each level, 24 hours after planting. The dose levels required a preliminary determination of a tissue culture toxicity. This was accomplished by adding logarithmic doses of the compound in saline to a series of tubes containing 5×10^5 cells/ml which were almost confluent. The cells were examined at 24, 48, and 72 hours. Any cytopathic effect (CPE) or inhibition of mitoses was scored as toxicity. Five more closely spaced dose levels were employed within the two logarithmic dosages, the higher of which showed toxicity and the lower no effect. The solvents used and the range finding data are presented in the toxicity data report under Results and Discussion. The dose level below the lowest toxic level was employed as the high level. Logarithmic dose levels were employed for the medium and low levels.

Cells were incubated at 37°C and examined twice daily to determine when an adequate number of mitoses were present. Cells were harvested by shaking when sufficient mitoses were observed, usually 24 - 48

hours after planting, centrifuged, and fixed in absolute methanol:glacial acetic acid (3:1) for 30 minutes.

The specimens were centrifuged, decanted, and suspended in acetic acid-orcein stain (2.0%) and a drop of suspension placed on a clean dry slide. Selected coverglasses 0.17 mm in thickness were placed on the suspension and the excess stain gently expressed from the slide. The coverglasses were sealed with clear nail polish and examined immediately.

The microscopes, objectives, oculars, filters and light sources were enumerated under the metaphase description. Positive controls used were TEM (at a concentration of 0.1 mcg/ml dissolved in saline) and negative controls which consisted of the vehicle in which the test compound was dissolved, which was 0.85% saline. Data were reported on forms currently used and expressed as percentages on the anaphase summary sheets.

3. Dominant Lethal Assay

In this test, male and female random bred rats from a closed colony were employed. These animals were 10-12 weeks old at the time of use. Ten male rats were assigned to each of 5 groups; 3 dose levels selected as described above, a positive control (triethylene melamine) (TEM) and a negative control (solvent only). The positive control was administered intraperitoneally. Administration of the test compound was orally by intubation in both the acute study (1 dose) and in the subacute study (1 dose per day for 5 days). Following treatment, the males were sequentially mated to 2 females per week for 8 weeks (7 weeks in the subacute study). Two virgin female rats were housed with a male for 5 days (Monday through Friday). These two females were removed and housed in a cage until killed. The male was rested on Saturday and Sunday and two new females introduced to the cage on

Monday. It has been our experience that conception has taken place in more than 90% of the females by Friday and that the two day rest is beneficial to the male as regards subsequent weekly matings. Females were killed using CO_2 at 14 days after separating from the male, and at necropsy the uterus was examined for deciduomata (early deaths), late fetal deaths and total implantations.

Sufficient animals were provided in our experimental design to accommodate for any reduction in the number of conceptions. Each male was mated with two females per week, and this provided for an adequate number of implantations per group per week (200 minimum) for negative controls, even if there was a fourfold reduction in fertility of implantations. Results were analyzed according to the statistical procedures described in Supplementary Materials and Methods. Corpora lutea, early fetal deaths, late fetal deaths and total implantations per uterine horn were recorded on the raw data sheets, which are submitted separately.

D. Supplementary Materials and Methods

1. Host-Mediated Assay In Vitro and Formulae

a. Bacterial in vitro plate tests

This method has been published by Ames: The Detection of Chemical Mutagens with Enteric Bacteria, in Chemical Mutagens; Principles and Methods for Their Detection, Vol. 1, Chapter 9, pp. 267-282, A. Hollaender, Editor, Plenum Press, New York (1971).

b. In vitro for mitotic recombination

(1) Strain D-3 was grown to stationary phase on complete medium agar plates at 30°C (3-4 days). Cells were rinsed from the plates and washed twice in saline and cell concentration determined spectro-

photometrically. (A standard curve previously determined for colony forming units versus % transmittance at 545 mu was easily used.)

(2) Cells from the concentration suspension were diluted appropriately into 0.067 M Phosphate buffer pH 7.2 to provide 5×10^7 cells/ml in a total of 25 ml.

(3) The test chemical was first tested for 4 hours at 30°C, with shaking, at concentrations which permitted determination of the 50% survival level. Then, if not included in the first experiment, the compound was tested again only at the 50% survival level. If 50% survival level could not be determined, the arbitrary test level of 5% w/v was used.

(4) Following treatment, cells were diluted and plated on complete agar medium for determination of total population and red sectors. Total surviving population was conveniently measured on plates of 10^{-4} and 10^{-5} dilutions using 0.2 ml per plate (5 plates), and sectors determined on plates of 10^{-3} and 10^{-4} dilutions using 0.2 ml per plate (5 plates). Plates were incubated for 2 days at 30°C followed by a holding period of 2 days at 4°C to promote color development with limited enlargement of the colonies. Red sectors were scored by systematically scanning the plates with a dissecting microscope at 10X magnification.

(5) The frequency of red sectors can then be calculated and may be expressed conveniently as sectors per 10^5 survivors for comparison with untreated controls.

(6) Ethyl Methane Sulfonate (EMS) was employed as the positive control in both in vitro systems.

c. Minimal medium (bacteria):

Spizizen's Minimal Medium:

4X Salt Solution:

$(NH_4)\ SO_4$	8.0 gm
K_2HPO_4	56.0 gm
KH_2PO_4	24.0 gm
Na Citrate	4.0 gm
$Mg\ SO_4$	0.8 gm
Biotin	0.004 gm
H_2O	qs to 1 liter Sterilize by autoclaving (121°C/15 min.)

Medium:

4X Salt Solution	:250 ml	
5.0% Glucose (sterile)	:100 ml	(If histidine is added at concentration of 30 mg/liter, this becomes a complete bacterial medium.)
1.5% Bacto-agar (sterile)	:650 ml	

d. Complete medium (bacteria):

Bacto-Tryptone	1.0 gm
Yeast-Extract	0.5 gm
Bacto-Agar	2.0 gm
Distilled H_2O	100.0 ml

Sterilize by autoclaving (121°C for 15 minutes).

e. Complete medium (yeast):

KH_2PO_4	1.5 gm
$MgSO_4$	0.5 gm
$(NH_4)_2SO_4$	4.5 gm

Peptone	3.5 gm
Yeast-Extract	5.0 gm
Glucose	20.0 gm
Agar	20.0 gm
Distilled H_2O	1000.0 ml

Sterilize by autoclaving (121°C for 15 minutes).

2. Cytogenetics In Vitro Preparation of Anaphase Chromosomes (from Nichols, 1970)

"Anaphase preparations may be made by several methods. One convenient approach is to grow cells directly on coverslips in petri dishes. With human fibroblasts 400,000 cells added to a 22 x 44 mm coverslip in a 50 mm petri dish grown in a 5% CO_2 atmosphere in air has proved very satisfactory. When adequate numbers of mitoses are visualized directly utilizing an inverted microscope (usually 48 to 92 hours after planting) the coverslip is transferred to absolute ethanol for 15 minutes for fixation. They are then stained with any one of a number of suitable stains (Fuelgen, May-Grunwald-Giemse, orcein) and attached to a slide with mounting media for evaluation. Anaphase preparations may also be prepared on cells grown in suspension or cells from a monolayer that have been put into suspension. In this instance the cells are centrifuged and fixed with the squash fixative. They are then suspended in the stain and a drop of the suspension put on the slide and covered with a coverslip. However, in this case, only the excess stain is gently expressed from under the coverslip and no squashing is carried out. In anaphase preparations no pretreatment with colchicine or hypotonic expansion is used and no technique for spreading the cells is used, so that the spindle and normal relationships of the chromosomes are not disturbed."

3. Statistical Analyses of Dominant Lethal Studies

The following statistical analyses were employed as a means of analyzing the results of the dominant lethal studies.

a. The fertility index

The number of pregnant females/number of mated females with the chi-square was used to compare each treatment to the control. Armitage's trend was used for linear proportions to test whether the fertility index was linearly related to arithmetic or log dose.

b. Total number of implantations

The t-test was used to determine significant differences between average number of implantations per pregnant female for each treatment compared to the control. Regression techniques were used to determine whether the average number of implantations per female was related to the arithmetic or log dose.

c. Total number of corpora lutea

The t-test was used to determine significant differences between average number of corpora lutea per pregnant female for each treatment compared to the control.

d. Preimplantation losses

Preimplantation losses were computed for each female by subtracting the number of implantations from the number of corpora lutea. Freeman-Tukey transformation was used on the preimplantation losses for each female and then the t-test was used to compare each treatment to control. Regression technique was used to determine whether the average number of preimplantation losses per female was related to the arithmetic or log dose.

e. Dead implants

Dead implants were treated the same as pre-implantation losses.

f. One or more dead implants

The proportion of females with one or more dead implants was computed, each treatment compared to control by chi-square test and Armitage's trend used for linear proportions to see if proportions were linearly related to either arithmetic or log dose. Also, probit regression analysis was used to determine whether the probit of the proportions was related to log dose.

g. Two or more dead implants

The proportion of females with two or more dead implants computed was treated same as above (f).

h. Dead implants per total implants

Dead implants per total implants were computed for each female and used Freeman-Tukey arc-sine transformation on data for each female; then used t-test to compare each treatment to control.

Historical control data was compiled on a continuous basis as studies were completed. In addition to comparing each treatment to control, as outlined above, each treatment was compared to a historical control.

In order to take variation between males into account, a nested model was used. An analysis of across weeks is also provided.

In addition to these tests, the distribution forms of the various parameters were tested in order to evaluate the appropriateness of some of the tests being used. Certain correlations between parameters may exist and were examined as one step to determine the appropriateness of models. If necessary, alternate test methods were implemented.

The results are presented in tabular form with the addition of historical control information. In addition to these tables, a written report of all findings is provided. As information became available from the on-going investigation of these data, it was reported and suggestions included for changes to the methods of analysis. The statistical reports give the level of significance using both a one-tailed and two-tailed test. Finally, a summary sheet for each study is provided.

MODEL

$$y_{ijk} = \mu + \alpha_i + c_{ij} + e_{ijk},$$

$i = 1, 2$ Group $\quad j = 1, 2, \ldots, 10$ Males within each group

$k = 1, 2$ Females within Males within Groups

ASSUMPTIONS: $\alpha_1 + \alpha_2 = 0$, $c_{ij} \sim nid(0, \sigma_c^2)$,

$e_{ijk} \sim nid(0, \sigma^2)$

Males are randomly drawn from infinite population

S.V.	d.f.	S.S.	M.S.	E(M.S.)	F
TOTAL	39	$\Sigma\Sigma\Sigma(y_{ijk} - \bar{y}_{...})^2$			
GROUPS	1	$20\Sigma(\bar{y}_{i..} - \bar{y}_{...})^2$	S_1^2	$\sigma^2 + 2\sigma_c^2 + 20\alpha_i^2$	S_1^2/S_2^2
MALES WITHIN GROUPS	18	$2\Sigma\Sigma(\bar{y}_{ij.} - \bar{y}_{i..})^2$	S_2^2	$\sigma^2 + 2\sigma_c^2$	S_2^2/S_3^2
REMAINDER	20	$\Sigma\Sigma\Sigma(y_{ijk} - \bar{y}_{ij.})^2$	S_3^2	σ^2	

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F. Abbreviations

1. mu = micron
2. mcg = ug = microgram
3. g = gram
4. kg = kilogram
5. ml = milliliter
6. rpm = revolutions per minute
7. °C = degrees centigrade
8. pH = power of the hydrogen ion concentration to the base 10
9. M = molar solution
10. conc. = concentration
11. MTD = maximum tolerated dosage = High = LD_5 if determined or else exceedingly high dose, such as 5 g/kg
12. INT = intermediate = medium level
13. USE = usage level if known = low level
14. BSS = balanced salt solution
15. C-metaphase = cells arrested in metaphase, using colchine or colcemid
16. LD_{50} = that dosage which produced 50% mortality in the group of animals treated
17. LD_5 = that dosage which produced 5% mortality in the group of animals treated
18. NC = negative control
19. PC = positive control
20. AU = acute usage level (low level)
21. AI = acute intermediate level (medium level)
22. AMTD = acute maximum tolerated dose level (LD_5 level, high level)

23. SAU = subacute usage level (low level)

24. SAI = subacute intermediate level (medium level)

25. SA LD_5 = subacute LD_5 level (MTD level, high level)

26. CO_2 = carbon dioxide

27. DMN = Dimethyl nitrosamine

28. EMS = Ethyl methane sulfonate

29. TEM = Triethylene melamine

30. DMSO = Dimethyl sulfoxide

31. MEM = minimal essential medium (Eagle's)

32. CPE = cytopathic effect

33. his = histidine marker

34. D-3 = mitotic recombinant strain of Saccharomyces

35. mf = mean mutant frequency

36. MFt/MFc = mean mutant frequency of the test compound group compared to mean mutant frequency of the negative control group

37. CFU = colony forming units

38. WI-38 = code name for a strain of human embryonic lung tissue culture cells

39. Rec x 10^5 = mitotic recombinants x 10^5

40. Mean B/A = mean frequency

41. tot. scr. = total scored

42. tot. = total

43. X^2 = a test of variation in the data from the computed regression line - tested in these studies at the 5% level

44. Aber. = aberrations

45. Frag. = fragment

46. HMA = host-mediated assay